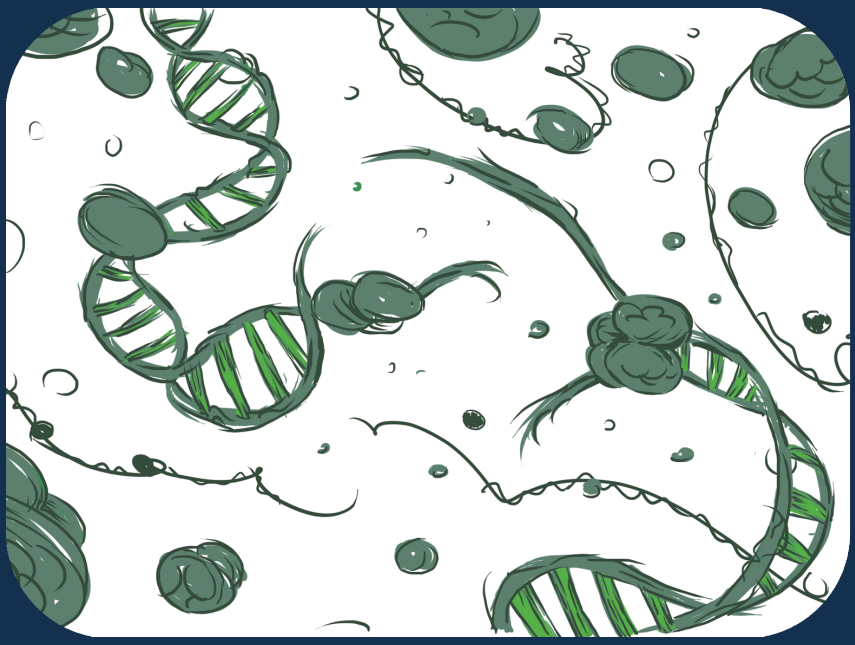
Measuring chromosome-end fusions in fission yeast

Hugo Almeida



Dissertation presented to obtain the Ph.D degree in Molecular Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
June, 2013

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Declaração

Esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Setembro de 2006 e Maio de 2012 no laboratório do Doutor Miguel Godinho Ferreira, Instituto Gulbenkian de Ciência em Oeiras Portugal no âmbito do Programa Doutoral do Instituto Gulbenkain de Ciência PDIGC2006. Todas as colaborações estão indicadas nas secções de 'Acknowledgments' e de 'Materials and Methods'.

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The process of data gathering that culminated in the production of this volume constituted an exquisite experience that is worthy of contemplation. For an aesthetic approximation of this experience, it is advised to accompany the reading of the ensuing chapters with the audition of John Zorn's *Goetia*, followed by The Locust's full-length album *Plague Soundscapes*.

In this narrative, the author plays both the roles of martyr and Devil.

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Summary

The ends of eukaryotic chromosomes are protected from illegitimate repair by structures called telomeres. These are comprised of specific DNA repeats bound by a specialized protein complex. When telomere function is compromised, chromosome ends fuse, generating chromosomal abnormalities and genomic instability.

I established a positive selection assay for capturing chromosome-end fusions in the fission yeast *Schizossacharomyces pombe*. A linear plasmid containing telomere sequences at each end was constructed. If the ends of this plasmid join, a genetic marker is expressed, thus capturing fusions by direct selection.

When introduced in wild-type cells, this plasmid becomes a stable linear episome that is unable to express the marker gene. To test the ability to capture telomere fusions, this plasmid was introduced in cells deficient for telomerase, an essential component of telomere maintenance. As predicted, fusions were detected after telomere dysfunction. In addition, specific subtelomeric homologies were repeatedly present at the fusion junctions. Concomitantly, these end-joining reactions require Rad16, an essential component of the single-strand annealing (SSA) repair pathway.

Their sequence suggests that they could be involved in generating hairpin structures, possibly stalling further DNA erosion before fusing. In agreement with this hypothesis, these fusions require the Rad32-Rad50-Nbs1 (MRN) complex and the endonuclease Ctp1, which are known to process DNA hairpins.

Surprisingly, this novel assay detected stochastic telomere-to-telomere fusions occurring in unperturbed cells. In contrast to the ones captured in telomerase mutants, these fusions were dependent on the non-

homologous end-joining repair pathway. In this dissertation, I propose that these fusions occur as a consequence of momentary de-protection at the ends of chromosomes. During replication, telomeres go through significant alterations in structure and telomeric protein content, which could open telomeres to DNA repair. Fusion events similar to the ones present in this system could cause genomic instability, sufficient to generate carcinogenesis.

Sumário

As pontas dos cromossomas eucariotas são protegidas da reparação de DNA indevida por estruturas chamadas telómeros. Os telómeros são constituídos por sequências específicas de DNA repetitivo e por um complexo proteico especializado. Quando a integridade dos telómeros é comprometida, por exemplo, por ausência de componentes específicos, as pontas dos cromossomas ficam desprotegidas. Nestas circunstâncias, as pontas dos cromossomas fundem, gerando anomalias cromossómicas e instabilidade genómica.

Como projecto de doutoramento, estabeleci um ensaio que captura fusões de cromossomas através de selecção positiva, na levedura de fissão *Schizossacharomyces pombe*. Um plasmídeo linear foi construído, contendo sequências teloméricas em cada ponta. Quando as pontas do plasmídeo se fundem, um gene marcador é expresso, permitindo assim a selecção directa de fusões.

Quando introduzido em células selvagens, este plasmídeo é estável na sua forma linear. O plasmídeo foi introduzido em células deficientes para a telomerase, uma proteína essencial para a manutenção dos telómeros, para testar a sua capacidade em capturar fusões de telómeros. Como previsto, a expressão do gene marcador foi detectada após fusão das pontas do plasmídeo. Estas fusões ocorrem sistematicamente após a erosão completa das sequências teloméricas. Adicionalmente, são mediadas por micro-homologias normalmente presentes nas regiões subteloméricas dos cromossomas. Confirmando estes resultados, estas fusões são dependentes de Rad16, componente da via de reparação de

DNA dependente de micro-homologias, chamadas de *single-strand annealing* (SSA). A sequência destas micro-homologias sugere que estas poderão gerar estruturas secundárias no DNA, prevenindo a degradação completa das pontas de DNA desprotegidas. Esta suspeita é reforçada pela descoberta de que estas fusões são dependentes do complexo Rad32-Rad50-Nbs1 (MRN) e da endonuclease Ctp1, que estão envolvidos no processamento de estruturas de DNA secundárias.

Inesperadamente, este ensaio também detectou fusões espontâneas entre telómeros em células sem perturbações. Ao contrário das fusões encontradas em mutantes de telomerase, estas fusões são dependentes de uma via de reparação de DNA independente de homologias, chamada *non-homologous end-joining* (NHEJ). Nesta dissertação, sugiro que estas fusões são consequência de desprotecção momentânea em telómeros funcionais. Sabe-se que os telómeros são sujeitos a alterações durante a replicação, que possivelmente disponibilizam as pontas dos cromossomas à maquinaria de reparação de DNA.

Estes tipo de fusões espontâneas geram instabilidade genómica, que em mamíferos poderão ser suficientes para gerar eventos carcinogénicos.

List of abbreviations

ALT – Alternative Lengthening of Telomeres, a recombination-based pathway of telomere maintenance

At (prefix) – designates a gene found in *Arabidopsis thaliana*

BFB – Breakage-Fusion-Bridge cycle

DDR – DNA Damage Response

DSB – Double-Strand Break

dsDNA – double-stranded DNA

h (prefix) – designates a gene found in humans

HR – Homologous Recombination

Ku - Non-Homologous End-Joining complex composed of Ku70 and Ku80 subunits

m (prefix) – designates a gene found in mouse

MMEJ – Microhomology-Mediated End-Joining

MRN – DNA Damage Response protein complex composed of Mre11(Rad32)/Rad50/Nbs1

NHEJ – Non-Homologous End-Joining

Sc (prefix) – designates a gene found in *Sachharomyces cerevisiae*

SSA – Single-Strand Annealing

ssDNA – single-stranded DNA

taz1*$^{+}$ *o/e – strain overexpressing Taz1 by integration of the pREP42-*taz1*$^{+}$ cassette into the *taz1*$^{+}$ locus

TPE – Telomere Position Effect, characterized by the inhibition of transcription near telomeres

Chapter 1 - General Introduction

1.1 - Brief considerations on the Cell Cycle

Cell growth and division are achieved through a tightly controlled sequence of events called the cell cycle. It guides the cell first into duplicating its contents and then dividing in two daughter cells, ensuring the genetic content of the parent cell is accurately replicated and transmitted to the next generation.

The cell cycle has been divided into two major functional stages: Interphase and Mitosis. Interphase is the stage where the majority of cell growth occurs and can be further separated into G_1, S, and G_2 phases. DNA replication occurs during S phase (S for synthesis). G_1 and G_2 are gap phases in which cell growth and protein synthesis take place. Progression of these phases is controlled via molecular pathways called checkpoints. Checkpoints arrest the cell cycle by inhibiting specific kinases called cyclin-dependent protein kinases, or Cdks (Elledge, 1996). With another class of proteins called cyclins, Cdks function as switches between cell cycle phases. Their inhibition allows more time to correct defects that could potentially hinder cell survival. G_1 and G_2 checkpoints ensure that protein and organelle content is suitable for faithful DNA replication and equal segregation of sister chromatids (Alberts et al., 2002).

G_1 progresses into DNA replication, through a point of no return known as Start in yeast, and Restriction Point in mammals (Novák et al., 2003). Yeast cells deprived of nutrients for two rounds of cell division, particularly from nitrogen sources, will arrest at Start. At Start, cells are capable of engaging in sexual reproduction by mating (fusing) with a cell of an opposing mating type. In the case of fission yeast, if mating partners are unavailable, cells will respond to poor nutrient conditions by entering into a specialized metabolic state known as $G_{0,}$ in which mating is no longer possible. This state allows cells to remain viable for months until favorable

environmental conditions are met once again, at which point they may resume growth (Yanagida, 2009).

After Start, the cell is fully committed to cell division, and competing processes such as mating and meiosis can no longer take place (Alberts et al., 2002; Nurse, 1985). After replication, the G_2 checkpoint controls entry into mitosis, in which the cell goes through a series of irreversible events that begin with nuclear division. Although most studied organisms follow these rules, fission yeast is a special case, in that it possesses no discernible G_1 phase, and spends most of its cell cycle in G_2. To force fission yeast cells to undergo a G_1-like phase, they can be manipulated into a dormant G_0 phase by subjecting them to nitrogen starvation (Su et al., 1996).

At the onset of Mitosis (or M phase), the two copies of each chromosome are bound as sister chromatids. The main purpose of M phase is to guarantee equal segregation of the chromosomes after they were duplicated during S phase, so that each daughter cell receives an identical copy of the parental genome (Alberts et al., 2002). In fission yeast, chromosomes remain attached to the Spindle Pole Body (the yeast's functional equivalent to the centrosome) during G_2 phase (Funabiki et al., 1993). Transition to M phase is marked by disruption of this attachment and equal segregation of chromatids to the poles (Funabiki et al., 1993).

Cell division in fission yeast is done through medial fission (Moseley and Nurse, 2009). Its life cycle is mainly haploid, but it will cease division and engage in sexual reproduction when nutrients, in particular nitrogen, become scarce (Klar, 1990). After sexual reproduction, zygotes may immediately enter meiosis (Davey, 1998). Meiosis generates a structure called an ascus, containing tetrads of 4 spores. Spores are resistant forms that allow yeast to survive under conditions of nutrient deprivation. Cells can remain in this quiescent state for a long period of time, until

environmental conditions are favorable for germination. When the spore germinates, fission yeast resumes its haploid cell cycle.

1.2 - The DNA Damage Response

DNA damage is a threat to the cell's survival. It can result in severe loss of genetic information that compromises viability and promotes rearrangements that may drive cancer (Finn et al., 2012). The origin of DNA damage is varied. It can result from external insults, such as radiation and chemical agents that interact with DNA (Finn et al., 2012; Zegerman and Diffley, 2009). In addition, it is also a regular outcome of cellular processes. In particular, a DNA double-strand break (DSB) may arise from meiotic recombination, DNA replication or reactive oxygen species produced endogenously (Branzei and Foiani, 2005; Raji and Hartsuiker, 2006; Wyman and Kanaar, 2006). To cope with the ubiquity of DNA damage, cells possess sophisticated DNA repair mechanisms that promptly respond to many different types of genetic injuries.

A DDR is a process that occurs in a regulated sequence of events to ensure that DNA lesions do not result in genomic catastrophe and cell death. DNA damage exists in many forms, including single base damage, inter- and intra-strand cross linking and single- and double-strand DNA breaks (Raji and Hartsuiker, 2006). A DDR broadly involves four steps: DNA damage recognition, checkpoint activation, repair of DNA damage and resumption of the cell cycle (Lisby and Rothstein, 2004). If an error is identified, such as a DNA DSB, checkpoints are activated and arrest cell cycle progression, allowing time for repair. If DNA lesions are left unchecked they can lead to loss of heterozigosity, improper transmission of genetic content, genomic rearrangements or an abortive cell division. Any of these events can lead to cell death or cancer (Raji and Hartsuiker, 2006).

The DDR has been extensively studied in *S. cerevisiae* (Finn et al., 2012). Studies in other organisms, including *S. pombe*, have highlighted that these are evolutionarily conserved processes and added new insights to the subject (Raji and Hartsuiker, 2006).

A DDR includes many regulators in a complex signaling cascade. These regulators can be grouped into three classes: sensors, mediators (or adaptors), and effectors (or transducer kinases; (Cobb et al., 2004; Kastan and Bartek, 2004). The success of the DDR relies in the amplification of the initial DNA damage signal from the sensors to the effectors. In the process, the DNA repair machinery reverses DNA damage while checkpoints inhibit cell cycle progression. In multicellular organisms, cells with irreparable amounts of DNA damage are driven to controlled cell death (Lydall, 2009).

When a DDR response is activated, DNA DSB repair will follow one of essentially two competing pathways. These are recombination-based methods of repair, such as Homologous Recombination (HR), or direct ligation of DNA breaks, such as Non Homologous End-Joining (NHEJ;(Ferreira and Cooper, 2004; Prudden et al., 2003). Choice of repair pathway depends on the nature of the DNA damage and cell cycle stage.

This section will succinctly describe some of the repair pathways involved in the response to DNA damage.

1.2.1 - Homologous Recombination

HR is a DNA repair pathway whereby DSB-interrupted DNA invades a homologous sequence and uses it as a template to copy the missing information. HR is also required for meiotic recombination (Raji and Hartsuiker, 2006). It is considered an error-free repair mechanism, since the template is usually a sister chromatid and no DNA sequences are lost or altered in the process (Kadyk and Hartwell, 1992). HR is mostly

restricted to the S/G_2 phases of the cell cycle (Aylon et al., 2004; Buis et al., 2012; Caspari, 2002; Chen et al., 1997; Chen et al., 2008; Ferreira and Cooper, 2004; Hinz et al., 2005; Huertas et al., 2008; Johnson and Jasin, 2000; Limbo et al., 2007; Rothkamm et al., 2003; Takata et al., 1998; Yu and Chen, 2004). It is thought that restricting HR to G_2 phase may reflect the availability of sister chromatids to act as error-free templates (Caspari, 2002; Ferreira and Cooper, 2004). Because fission yeast is a haploid organism there is no homologous template available in G_1. This further prevents the possibility of error-free HR repair during this phase.

The main sensor component for HR is the MRN complex (Finn et al., 2012; Willis and Rhind, 2010). In fission yeast, MRN is composed of three subunits: Rad32, Rad50 and Nbs1. The complex is tethered to broken DNA ends by its Nbs1 subunit and dimerizes through its Rad50 subunit, bridging two broken DNA ends together (de Jager et al., 2001; Hopfner et al., 2002; Williams et al., 2009; Williams et al., 2008). In fission yeast, recognition of DNA DSBs by MRN is performed in association with Ctp1 and the checkpoint kinase Tel1 (Akamatsu et al., 2008; Carson et al., 2003; Chahwan et al., 2003; Finn et al., 2012; Limbo et al., 2007; Lisby et al., 2004; Nakada et al., 2003; You et al., 2005; Yu, 2006; Yu and Chen, 2004).

HR initiation requires that DNA DSBs be subjected to 5'-to-3' resection, generating a 3'-overhang (Akamatsu et al., 2008; Hartsuiker et al., 2009; Langerak et al., 2011; Limbo et al., 2007; Shim et al., 2010; Wyman and Kanaar, 2006). Experiments in both yeasts and human cells show that resection is promoted by MRN and Ctp1, along with additional nucleases such as ExoI (Cejka et al., 2010; Clerici et al., 2005; Langerak et al., 2011; Limbo et al., 2007; Mimitou and Symington, 2008; Nicolette et al., 2010; Sartori et al., 2007; Shim et al., 2010; Williams et al., 2009; You et al., 2009; Zhu et al., 2008). In budding yeast, it has been proposed that the

Rad32 orthologue Mre11 acts directly on DNA end resection with the aid of Ctp1 orthologue Sae2 (Garcia et al., 2011). Analysis of meiotic recombination intermediates shows that Mre11 nicks the DNA upstream of the 5'-strand end. Subsequently, Mre11 performs its 3'-to-5' nuclease activity to create a 3'-overhang in conjunction with Sae2's 5'-to-3' nuclease activity.

Generation of a 3'-overhang at DNA DSBs paves the way for Replication Protein A (RPA). RPA binds specifically to single-stranded DNA. This inhibits 3'-ssDNA degradation, while promoting further 5'-to-3' degradation. RPA also recruits additional checkpoint components, including the checkpoint kinase Rad3 (Limbo et al., 2011; Zou and Elledge, 2003). In fission yeast, this kinase, along with Tel1, activates effector kinases Chk1 and Cds1. These effector kinases induce a cell cycle arrest by inhibiting cyclin activity.

The 3'-overhang is also recognized by the ssDNA-binding recombinase Rad22. Along with other recombinases such as Rhp51, Rad22 finds homologous sequences to serve as template for HR (Muris et al., 1993; Muris et al., 1997; Ozenberger and Roeder, 1991; Sung, 1994; Symington, 2002; Wyman and Kanaar, 2006).

Thus, HR is an error free DNA repair pathway. However, because it requires the presence of a sister chromatid as a template for repair, its activity is usually restricted to S/G_2 phase.

1.2.2 - Non Homologous End-Joining

NHEJ is a DNA repair pathway that involves the tethering and fusion of two unprotected DNA ends, independently of homology (Gu et al., 2007a; Gu et al., 2007b). A particularly well-known NHEJ event is the ligation of DNA during V(D)J recombination (Malu et al., 2012). V(D)J

recombination is responsible for the generation of genetic variability found in antigen receptors of mammalian lymphocytes.

Contrary to HR, mammalian and budding yeast NHEJ, fission yeast NHEJ is proficient even in the absence of several components of the DNA damage and replication checkpoints (Callén et al., 2009; Chen et al., 2012a; Manolis et al., 2001; Martin et al., 1999; Tomimatsu et al., 2009). Thus, NHEJ does not seem to require checkpoint activation.

The precision of NHEJ repair has been a debated matter. NHEJ has been reported to result in deletions at the intervening breakpoints, as DSBs may be subjected to degradation prior to successful DNA ligation (Goedecke et al., 1994; Tseng et al., 2008; Wilson and Lieber, 1999). However, some studies suggest that the majority of NHEJ repair events in fission and budding yeast result in error-free end-joining reactions (Boulton and Jackson, 1996a, b; Li et al., 2012). In fission yeast, plasmid ligation assays showed that 40% of blunt-ended breaks are repaired precisely, while cohesive breaks lead only to 10% of precise ligations (Manolis et al., 2001). In mammalian cells, only end-joining repair of cohesive ends is error-free (Guirouilh-Barbat et al., 2004). Thus, the published data suggests that the precision of NHEJ depends on the conditions provided by the DNA DSBs. Additionally, since this method of repair does not require homology between the intervening DNA sequences, it may cause genomic rearrangements (Guirouilh-Barbat et al., 2004; Lieber, 2010). For these reasons, NHEJ is usually considered an error-prone mechanism of DNA repair (Lieber, 2010).

Nevertheless, NHEJ is the preferred method of repair in G_1 phase for haploid and diploid organisms (Ferreira and Cooper, 2004; Lieber, 2008). Furthermore, the use of NHEJ during G_1 phase prevents loss of heterozygosity that would rise through the use of HR between homologous

chromosomes (Moynahan and Jasin, 1997). This suggests that NHEJ is the most advantageous repair pathway during G_1 phase.

There are 3 essential NHEJ components known in fission yeast: the Ku complex, DNA ligase Lig4 and Xlf1 (Cavero et al., 2007; Hentges et al., 2006; Li et al., 2012; Manolis et al., 2001). In contrast to HR, NHEJ is an iterative process and does not require a precise order of events (Lieber, 2010). In addition, depending on the type of DSBs, NHEJ may require other activities such as ssDNA gap-filling or DNA flap removal at the fusion junction. Hence, other components such as DNA polymerase Pol4 and several nucleases may be recruited for NHEJ reactions (Chen and Kolodner, 1999; Daley, 2005; Daley et al., 2005; Haber, 1998; Li et al., 2012; Ma et al., 2002; Tseng et al., 2008; Wilson and Lieber, 1999). These events may occur before or after ligation, which makes NHEJ a highly versatile repair pathway, able to adapt to different kinds of DNA damage.

The Ku complex is thought to be the first component of the canonical NHEJ repair pathway to recognize and interact with DNA DSBs (Lieber, 2010). The Ku complex is a heterodimer composed of Ku70 and Ku80 (Baumann and Cech, 2000; Boulton and Jackson, 1996a, b; Miyoshi et al., 2003; Taccioli et al., 1994). It possesses high affinity for dsDNA and tethers DNA DSBs (Mimori and Hardin, 1986; Paillard and Strauss, 1991). Although all of the NHEJ components can bind to DSBs independently, it is thought that Ku recruits the remaining NHEJ components that process and ligate DNA breaks (Grundy et al., 2013; Hentges et al., 2006; Lieber, 2010; Yano et al., 2007).

In budding yeast, the ligation reaction is performed by a complex including ScLig4 and ScLif1, which serves to stabilize ScLig4 (Boulton and Jackson, 1998; Herrmann et al., 1998; Teo and Jackson, 1997; Wilson et al., 1997). In addition, NHEJ becomes defective in the absence of ScNej1, orthologue of fission yeast Xlf1 (Frank-Vaillant and Marcand, 2001; Liti and

Louis, 2003; Wilson, 2002). ScNej1 is therefore considered a regulator of NHEJ. In budding yeast, absence of ScMre11 or ScRad50 disrupts NHEJ (Chen and Kolodner, 1999; Daley et al., 2005; Haber, 1998). In NHEJ, it is thought that MRX bridges DNA ends, promoting ScLig4-mediated ligation (Chen et al., 2001; D'Amours and Jackson, 2002). In contrast, plasmid repair assays in fission yeast show that MRN is largely dispensable for NHEJ (Manolis et al., 2001; Wilson et al., 1999). However, another study in fission yeast observes that MRN is required for NHEJ reactions that involve the capture of mitochondrial DNA at fusion junctions (Decottignies, 2005). In addition, MRN is also required for NHEJ-mediated telomere fusions (Correia Reis et al., 2012). Thus, fission yeast MRN may be required for specific types of NHEJ reactions. Like in fission yeast, MRN does not seem to be required for NHEJ in humans (Di Virgilio and Gautier, 2005).

In humans, NHEJ is the dominant pathway for DNA repair (Lieber, 2010). Human Ku, along with protein kinase DNA-PKcs (DNA-PK catalytic subunit), forms a larger complex called DNA-PK (Blunt et al., 1995; Finnie et al., 1995; Jackson and Jeggo, 1995; Taccioli et al., 1994; Yaneva et al., 1997). Absence of either results in defective NHEJ. There is evidence that DNA-PK is also involved in the recognition of DSBs and initiation of a DDR, in a manner that is redundant with ATM (Callén et al., 2009; Chen et al., 2012a; Tomimatsu et al., 2009). While mammalian NHEJ repair does not seem to depend on additional DSB sensors (Quennet et al., 2011), it is possible that checkpoint activation is a necessary step in mammalian NHEJ repair via DNA-PKcs. Human DNA Ligase IV also forms a complex with XRCC4, the orthologue of budding yeast ScLif1 (Critchlow et al., 1997; Grawunder et al., 1997; Li et al., 1995; Lieber, 2010; Sibanda et al., 2001). Both are required for proper ligation of broken DNA ends. Their *in vitro* activity is stimulated by hXLF, orthologue of fission yeast Xlf1/budding yeast Nej1 (Gu et al., 2007b; Hentges et al., 2006). Simultaneously, hXLF's

stability at DSBs depends on XRCC4 (Ahnesorg et al., 2006; Callebaut et al., 2006).

Current knowledge of DNA repair pathways suggests that HR and NHEJ regulate each other (Chapman et al., 2012). Because Ku has low affinity for ssDNA, generation of long 3'-overhangs at DSBs favors HR repair over NHEJ. Presumably, the Mre11 subunit of MRN inhibits Ku binding at DSBs by promoting 5'-strand resection (Langerak et al., 2011). The reverse is also true, as Ku binding to DSBs inhibits HR by blocking 5'-end resection (Chapman et al., 2012).

In conclusion, NHEJ is a versatile mechanism of DNA repair that involves direct ligation of DNA ends, independently of homology.

The next section describes other types of DNA end-joining mechanisms. In contrast to NHEJ, these mechanisms are homology-dependent.

1.2.3 - Microhomology mediated mechanisms of end-joining

By disrupting the NHEJ pathway, additional mechanisms of DNA end-joining repair were uncovered (Bentley, 2004; Boulton and Jackson, 1996b; Decottignies, 2007; Feldmann et al., 2000; Guirouilh-Barbat et al., 2004; Heacock et al., 2004; Ma et al., 2003; Manolis et al., 2001; Yu and Gabriel, 2003). These are DNA end-joining pathways that depend on the annealing of microhomologies. These microhomologies can vary from very small (3-10 bp) perfectly complementary sequences up to several hundred base pairs of homology with mismatches (Decottignies, 2007; Ma et al., 2003; Pâques and Haber, 1999; Wang and Baumann, 2008). In order for microhomologies to participate in end-joining reactions, DSBs need to undergo 5'-end resection (Pâques and Haber, 1999). Since DSBs may not possess microhomologies at the ends, annealing may only occur several

kilobases away from the break. Consequently, these end-joining processes usually result in deletions that can span several kilobases (Decottignies, 2007; Pâques and Haber, 1999).

One of these repair mechanisms is called Single Strand Annealing (SSA). It is considered a variation of HR, since it is dependent on fission yeast Rad22/budding yeast Rad52, and independent of Ku (Decottignies, 2007; Pâques and Haber, 1999). In budding yeast, it is distinguished from other types of HR by its dependence on Rad59, independence of MRX and inhibition by Rad51 (Davis and Symington, 2004; Ira and Haber, 2002; Ivanov et al., 1996; Pâques and Haber, 1999; Sugawara et al., 2000; Symington, 2002). Plasmid repair assays in budding yeast show that, in some instances, SSA is preferred over canonical HR (Pâques and Haber, 1999). SSA's efficiency is unrelated to the physical distance of the DNA breaks involved, since it is able to ligate HO-induced DSBs from different chromosomes as efficiently as it ligates DSBs in the same chromosome.

In addition to SSA, specific microhomology-mediated end-joining (MMEJ) mechanisms were uncovered in budding yeast. Like SSA, MMEJ is dependent on Rad59, but inhibited by Rad51 (Villarreal et al., 2012). In addition, MMEJ is frequently MRX-dependent, but Ku- and Rad52-independent (Ma et al., 2003; Ozenberger and Roeder, 1991; Yu and Gabriel, 2003). However, this is not true for all instances of MMEJ. Length and distance of microhomologies from the break seem to determine which specific MMEJ pathway is used for repair (Villarreal et al., 2012). Budding yeast Rad52 promotes microhomology-mediated repair at DSBs with 15-18 bp of microhomologies, while inhibiting this type of repair at DSBs with smaller (12-13 bp) microhomologies (Villarreal et al., 2012). Concomitantly, the efficiency of MMEJ is inversely proportional to the distance between the homologies and the break, and increases with the size of the microhomologies (Ozenberger and Roeder, 1991). For completion, it

requires additional components responsible for removing surplus 3'-DNA flaps from the fusion junction, such as the Rad1/Rad10 complex (Carr et al., 1994; Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Sugawara et al., 1997; Villarreal et al., 2012). Thus, it is reasonable to suggest that the distance and size of microhomologies involved in repair could dictate which end-joining mechanisms intervene.

In fission yeast, MMEJ is dependent on Rad22, and inhibited by Ku, concomitant with HR-related pathways (Decottignies, 2007). For this reason, it has been suggested that fission yeast MMEJ is SSA at very short microhomologies, of at least 5 bp. This pathway is also dependent on the exonuclease Exo1. Furthermore, its efficiency is severely reduced in the absence of DNA repair components such as the *RAD1* orthologue *rad16*$^{+}$, and the MRN component Rad50. In opposition to NHEJ, MMEJ activity is more prevalent during G_2.

In mammalian cells, MMEJ is a major pathway of repair for non-cohesive DNA ends (Guirouilh-Barbat et al., 2004). It is usually termed Alternative Non Homologous End-Joining (A-NHEJ), because it joins DSBs in a Ku and Lig4-independent manner. A-NHEJ is also dependent of MRN and DNA ligases Lig1 and Lig3 (Audebert et al., 2004; Corneo et al., 2007; Deriano et al., 2009; Simsek et al., 2011; Wang et al., 2005; Xie et al., 2009; Yan et al., 2007). Additionally, A-NHEJ is considered a category of HR, since it is partially dependent on Rad22 orthologue mRAD52. Furthermore, it is independent of XRCC4 and inhibited by KU. Like budding yeast's MMEJ, it is inhibited by mRAD51 (fission yeast's Rhp51). Mammalian MMEJ is reported to rely in microhomologies as small as 1 bp, as well as resulting in deletions at the fusion junction (Feldmann et al., 2000; Kabotyanski et al., 1998; Stark et al., 2004).

Thus, it seems that microhomology-mediated mechanisms of repair are HR-derived pathways which are able to adapt to different kinds of DSBs by employing different DNA repair components.

1.4.4 - Processing and repair of hairpin DNA structures

Some types of DNA breaks include aberrant structures, such as DNA-protein bonds (Hartsuiker et al., 2009) or secondary DNA structures (Paull and Gellert, 1998), that must be processed before repair can take place. Secondary DNA structures may take different conformations. For example, structures called G-quadruplexes may form in Guanine-rich sequences. These form stacked nucleic acid structures by establishing hydrogen bonds between guanine residues (Bochman et al., 2012). In addition, resection at DNA DSBs can expose ssDNA containing palindromic sequences. These are able to anneal with each other, generating non-canonical DNA structures. These palindromes are often separated by small stretches of non-complementary DNA, forming hairpin-like structures when annealed. In the genome, they can create recombinogenic cruciforms and lead to genomic amplifications if left unresolved (Lobachev et al., 2002; Rattray et al., 2001). Experiments in budding yeast have elucidated the mechanisms that involve hairpin removal. Mutants for any of the MRX components or for *ctp1*$^{+}$ orthologue SAE2 are unable to repair DNA hairpins (Lobachev et al., 2002; Rattray, 2005; Rattray et al., 2001). Sae2 has an endonuclease activity with a preference for hairpin structures, responsible for cutting ssDNA adjacent to hairpins (Lengsfeld et al., 2007). This activity is enhanced by MRX through its 3'-to-5' exonucleolytic activity. In addition, MRX has been implicated in cleaving the tips of hairpins (Paull and Gellert, 1998; Trujillo et al., 2003; Trujillo and Sung, 2001). This is a highly conserved function of the complex, since *E. coli*'s Rad50 homologue

SbcCD nuclease, as well as human MRN are also able to cleave hairpins at their tip (Connelly et al., 1998; Paull and Gellert, 1999). Human CtIP was also shown to function as a backup hairpin-clipping nuclease during processing of DSBs in V(D)J recombination (Helmink et al., 2011). Fission yeast's Rad32 and Rad50 were found to be required for the high recombination frequency found in a genomic palindrome (Farah et al., 2002), suggesting that the function is conserved in fission yeast.

This section discussed a variety of DNA repair mechanisms which may be activated at DNA DSBs. The following sections will describe specific DNA DSBs that exist in the eukaryotic genome: natural chromosome ends. These possess special properties, which normally protect them from DNA repair.

1.3 - An overview of telomere History

Telomeres are specialized structures at the ends of eukaryotic chromosomes entrusted with two main functions: protecting chromosome ends from being recognized as deleterious DNA breaks, and ensuring full replication of chromosome ends (de Lange, 2005). These are composed of repetitive G-rich sequences of DNA, which are species-specific, bound by a protein complex known as "Shelterin" (de Lange, 2005).

Our knowledge of telomeres dates back to seminal discoveries from the 1930's and 1940's by Barbara McClintock and Hermann Muller. These findings showed that chromosome ends were different from other chromosome breaks because they are protected. Barbara McClintock's experiments in maize demonstrated that, in contrast to normal chromosome ends, chromosome breaks would fuse to each other, generating dicentrics (McClintock, 1938, 1939). These cause segregation problems during mitosis, which are resolved through breaking of the chromosomes. These

breaks fuse once again, initiating a cycle of events that McClintock called a "breakage-fusion-bridge" (BFB) cycle (McClintock, 1941); Figure 1.1). This process generates genomic instability that results in duplication or loss of genetic information with each new cell division. It was Hermann Muller, however, that proposed to call "telomere" to the special structure at the ends of chromosomes, from the greek telos (τέλος), "end", and meros, (μέρος) "part" (Muller, 1938).

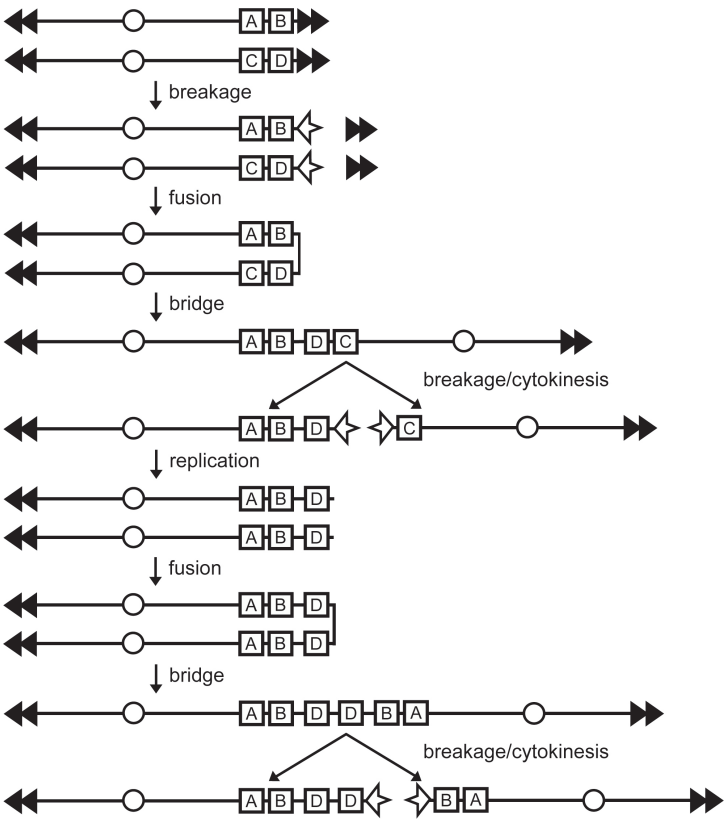


Figure 1.1 - The breakage-fusion-bridge cycle is an engine for genomic instability. Loss of telomere protection leads to chromosome fusions. The resulting dicentrics break during chromosome segregation. Breaks may occur outside of the original fusion junction, resulting in unequal genetic exchange between the intervening chromatids. The ensuing unprotected ends reinitiate the cycle. Black triangles represent telomeres. White circles represent centromeres. Adapted from Bailey and Murnane, 2006.

Further discoveries on DNA replication highlighted another problem for DNA ends. Semi-conservative DNA replication can only be performed in the 5' to 3' direction, using a template complementary DNA molecule (Sugino and Araki, 2006). Furthermore, DNA polymerases cannot initiate replication by themselves. Instead, DNA polymerases require an RNA primer to extend the new strand of DNA, called Okazaki fragment. As a consequence, the extremities of the 5'-end cannot be fully replicated, leaving a 3'-overhang when the Okazaki fragment is removed (Figure 1.2).

Continuous replication of the resulting DNA molecule results in incomplete 3'-end replication, leading to gradual loss of DNA ends. This became known as the "end-replication problem", and it was first raised by James Watson in 1972 (Watson, 1972), while studying T7 bacteriophage replication. However, formal demonstration of the end-replication problem was only performed *in vitro* in 2001 (Ohki et al., 2001).

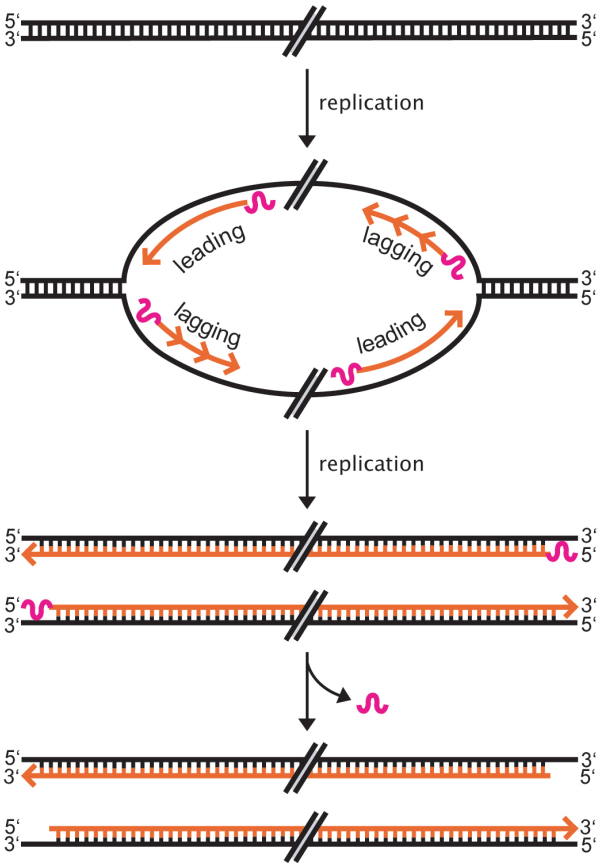


Figure 1.2. The end-replication problem. Semi-conservative DNA replication requires an RNA primer (Okazaki fragment) to initiate DNA replication. As a consequence, lagging strand replication cannot fully replicate its 5'-end, leaving a gap after the RNA primer is removed. Wavy pink lines represent Okazaki fragments. Parental DNA is indicated by black lines, and replicated DNA by orange lines. Adapted from Watson, 1972 and Lingner et al., 1995.

However, the authors left unexplained how chromosomes could be inherited and maintained throughout generations, if the ends were gradually shortening. The mystery began to unravel with the discovery of repetitive G-rich sequences at *Tetrahymena* telomeres by Elizabeth Blackburn and Joseph Gall (Blackburn and Gall, 1978). These *Tetrahymena* sequences were found to be able to stabilize the ends of linear plasmids in budding yeast (Dani and Zakian, 1983; Szostak and Blackburn, 1982), proving that they were at the basis of an evolutionarily conserved mechanism of chromosome-end protection. Additional experiments revealed that telomere

ends were maintained through the nontemplated addition of repeats by a telomere reverse transcriptase called telomerase, that would counteract incomplete replication of chromosomes (Blackburn and Greider, 1985; Greider and Blackburn, 1987; Shampay et al., 1984). This enzyme would add new repeats using an RNA template (Shippen-Lentz and Blackburn, 1990).

Previously, Leonard Hayflick's work revealed that cultured human cells did not proliferate indefinitely (Hayflick, 1965; Hayflick and Moorhead, 1961). Rather, cell cultures grow until a point where cell division no longer occurs, remaining in a cell cycle-arrested state, despite retaining metabolic viability. To this state he called senescence, and the number of cell divisions required to achieve it was later coined "the Hayflick limit". This discovery prompted Alexei Olovnikov to raise a possible relationship between the Hayflick limit and the end-replication problem (Olovnikov, 1973). The author suggested that gradual resection of DNA ends was the reason why cultured human cells could not proliferate indefinitely. In doing so, he established telomeres as the main contenders for setting a "mitotic clock" for cell proliferation and, consequently, as potential determinants of biological lifespan of an organism.

The connection between senescence and telomere shortening was later confirmed by Victoria Lundblad and Jack Szostak. Using a linear plasmid-based assay to screen for mutants with telomere phenotypes, the authors discovered *EST1* (Ever Shorter Telomeres). This gene encodes for a telomerase subunit that, when mutated, results in gradual erosion of telomeres, leading to senescence and chromosome loss (Lin and Zakian, 1995; Lundblad and Szostak, 1989). A similar correlation between telomere size and replicative potential was soon reproduced in human somatic cells, where telomeres shortened with increasing cell divisions (Allsopp et al., 1992; Harley et al., 1990). In agreement with these results, human somatic

tissue does not show any detectable levels of *in vitro* and *in vivo* telomere repeat addition (Counter et al., 1992; Kim et al., 1994). The impact of telomere shortening in the proliferative capacity of human cells was finally demonstrated when Bodnar and coworkers proved that human somatic cells, in the presence of telomerase, would elongate telomeres and avoid senescence (Bodnar, 1998). The same studies were able to identify telomerase activity in germline, immortal and cancer cell lines, further underlining the requirement of telomerase for continued cell proliferation.

As a consequence, telomeres and telomerase have been given much attention in cancer research. It has been shown that the large majority of cancers are able to proliferate indefinitely by re-activating telomerase (Hahn et al., 1999; Kim et al., 1994; Meyerson et al., 1997; Shay and Bacchetti, 1997). Normally, cells enter into senescence before reaching critical telomere shortening, thus avoiding genomic instability and death (Bodnar, 1998; Harley et al., 1990). However, if senescence is overcome, telomere dysfunction is conducive to chromosome instability such as the BFB cycles first observed by McClintock (Murnane, 2012). Coincidentally, cancer incidence increases with age, which is accompanied with telomere shortening (Bray et al., 2013). However, the role for telomerase in cancer progression remains inconclusive. Absence of functional telomerase in different mice models can inhibit or promote malignancy (Blasco et al., 1997; González-Suárez et al., 2000; Rudolph et al., 1999). It is presumed that the outcome depends on the tissues' propensity to develop telomerase-independent mechanisms of telomere maintenance (Hu et al., 2012). In parallel, constitutive overexpression of telomerase in mice resulted in a higher incidence of cancer (Artandi, 2002; Canela et al., 2004; González-Suárez et al., 2001). Thus, it is currently thought that, although telomere dysfunction breeds genomic instability required for malignancy, reactivation of telomerase or of telomerase-

independent mechanisms of telomere maintenance is an important step in the consolidation of carcinogenesis (Hu et al., 2012; Pereira and Ferreira, 2012). To test this hypothesis, the DePinho laboratory used a prostate cancer mouse model with an inducible copy of telomerase, so that its expression would only be activated after telomere dysfunction (Ding et al., 2012). In contrast to negative controls, telomerase-positive mice were prone to aggressive and invasive prostate tumors with genomic rearrangements.

The importance of telomeres for cell proliferation suggests that defects in telomere elongation would drastically reduce organismal lifespan. Accordingly, mutations in telomerase and other telomere components that lead to abnormal telomere shortening were reported in patients with premature aging syndromes such as dyskeratosis congenita and pulmonary fibrosis (Mitchell et al., 1999; Tsakiri et al., 2007; Yang et al., 2011).

Given this, telomere shortening has been proposed as a master regulator of aging and lifespan (Sahin and DePinho, 2010). Accordingly, late-generation telomerase mutant mice show several phenotypes such as decreased longevity, tissue atrophy, impaired wound healing and sterility, which seem to emulate the functional decline of tissues and organs characteristic of old age (Herrera et al., 1999; Lee et al., 1998; Rudolph et al., 1999). Recently, it was shown that the phenotypes of these telomerase mutants were not irreversible and could be partially rescued by transient telomerase expression, coinciding with telomere elongation (Bernardes de Jesus et al., 2012; Jaskelioff et al., 2011). It remains to be seen whether the same results are obtained in wild type (WT) old-age animals.

Understanding telomere maintenance and dysfunction is therefore of paramount importance for a deeper comprehension of the mechanisms underlying genomic stability, disease and aging.

1.4 - Telomeres are a tightly regulated structure comprised of DNA, proteins and non-coding RNA

Dissecting the mechanisms behind chromosome end protection is an ongoing enterprise in the telomere field. Much is already known, particularly in well characterized systems such as fission, budding yeast, mouse and human cell models. Since the work described in this dissertation was performed using fission yeast as a model, the following description of telomere components will focus mainly on what is known from this organism. Similarities and differences between this and other systems will be detailed whenever considered relevant. Comparisons will underlie an understanding of telomeres as an evolutionarily conserved mechanism of chromosome-end protection. Fission yeast's telomeres are remarkably similar in composition to its human counterpart (Figure 1.3; (Miyoshi et al., 2008). As such, it is a privileged unicellular model organism for the study of telomere genetics.

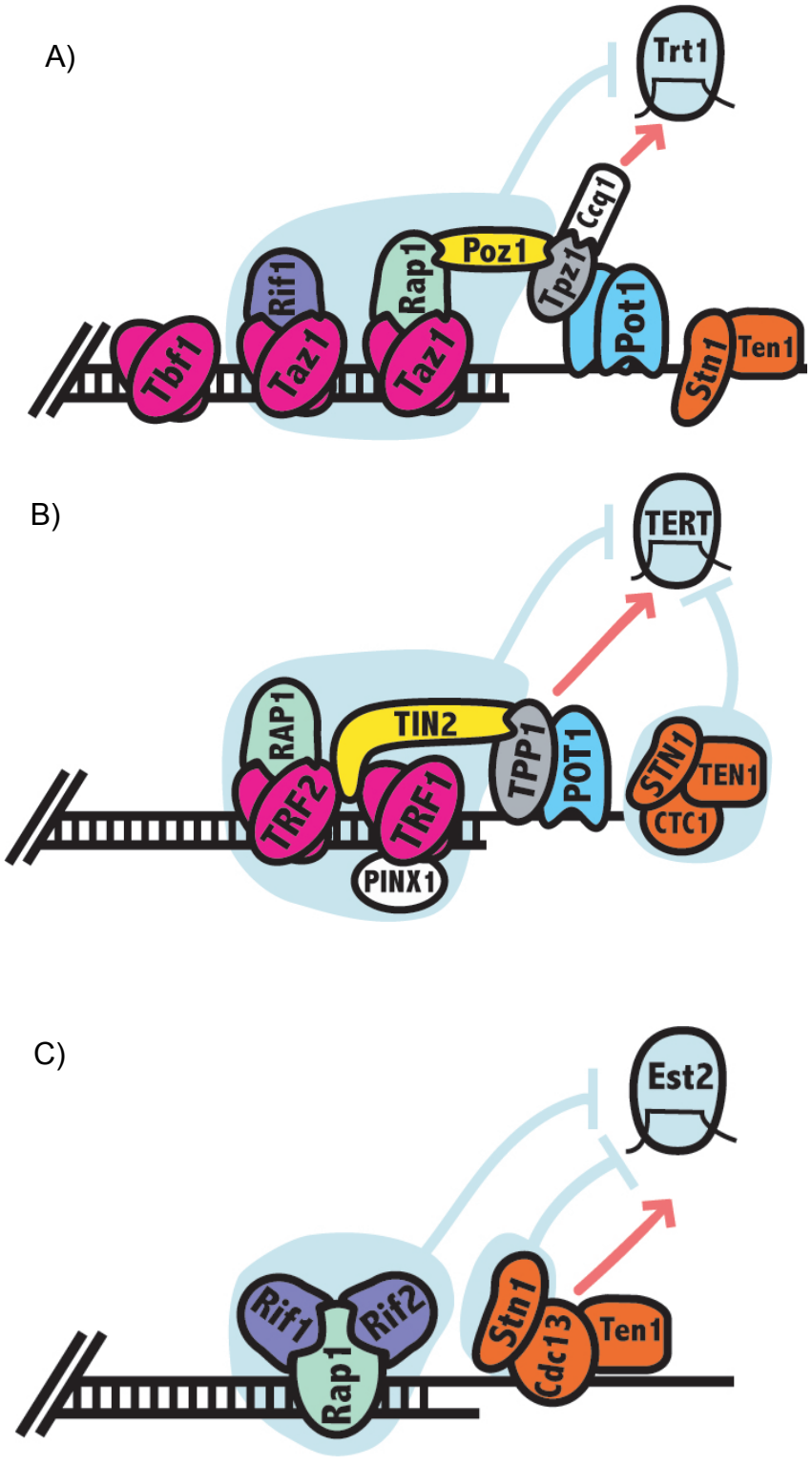

A)
Trt1
Ccq1
Tpz1
Poz1
Rap1
Rif1
Taz1
Taz1
Tbf1
Pot1
Stn1
Ten1
B)
TERT
RAP1
TIN2
TPP1
POT1
STN1
TEN1
CTC1
TRF2
TRF1
PINX1
C)
Est2
Rif1
Rif2
Rap1
Stn1
Cdc13
Ten1

Figure 1.3. Shelterin and other telomere components. A) Fission yeast telomeres, adapted from Myioshi et al., 2008; B) Human telomeres, adapted from Sfeir and de Lange, 2012; C) Budding yeast telomeres, adapted from Aurichect et al., 2008. Light blue areas indicate telomerase inhibitors. Red arrows indicate telomerase recruiters/activators.

In fission yeast, telomeres are around 300 bp in length (Matsumoto et al., 1987). Telomeric DNA is composed of repetitive sequences, of which the consensus is GGTTAC in this organism. In some organisms, like humans and *Tetrahymena*, the consensus sequence is repeated uniformly, corresponding to one and a half repeats (Collins, 2006). In fission yeast, however, repeats are interrupted by short spacers that vary in length and sequence, in a manner that could be summarized by the formula $G_nGGTTAC(A/AC)_{0-1}$ (Ares Jr and Chakrabarti, 2008). To explain this variability, it has been proposed that the end of the template in the telomerase RNA subunit (TER1) is not well defined in fission yeast. As a consequence, template translocation to the end of telomeres is subject to variability and slippage, causing the occasional replication of additional sequences (Leonardi et al., 2008; Webb and Zakian, 2007).

As a primer for telomere repeat addition, telomerase uses a 3' single-stranded (ss) G-rich DNA overhang, known as G-overhang, that protrudes from a double-stranded DNA telomere region (Blackburn and Greider, 1985; Henderson and Blackburn, 1989; Klobutcher et al., 1981; Wright et al., 1997). Its presence could be explained by the cell's inability to fully replicate the lagging strand, leaving a 3'-ssDNA region. However, the G-overhang is a conserved feature of telomeres and is actively maintained by enzymatic resection of the 5'-overhang (Deng et al., 2009a; Jacob et al., 2001; Makarov et al., 1997; Muñoz-Jordán et al., 2001; Wellinger et al., 1996; Wellinger et al., 1993b; Wu et al., 2012). Moreover, it is required for

the structural integrity of telomeres and binding of Shelterin (de Lange, 2005). Thus, Lingner and co-workers suggested a revision of the end-replication problem (Lingner et al., 1995). This revision accounts for the G-overhang functions in chromosome-end protection. The authors argue that the end-replication problem does not result from the inability of DNA polymerases to synthesize the 5'-ends. Instead, it results from the inability to completely re-synthesize the G-overhangs (Figure 1.4). Because there is no template for the G-overhang, these need to be replenished in a non-templated manner, by telomerase.

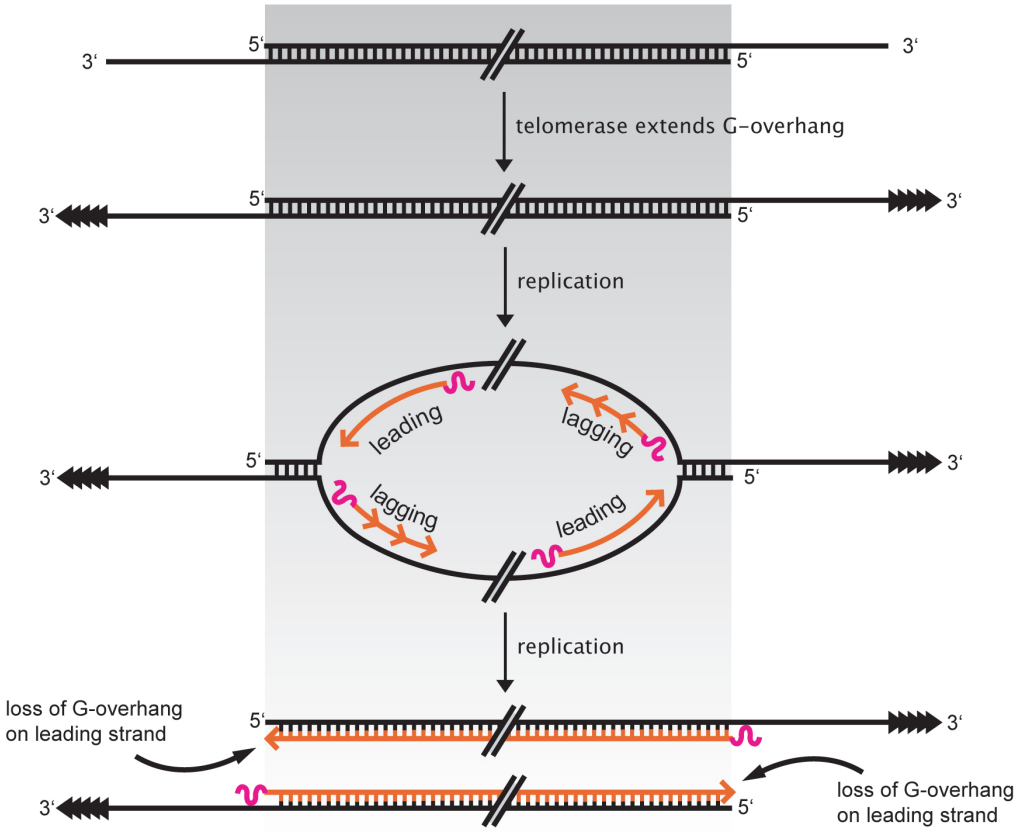


Figure 1.4. The revised end-replication problem. Telomere repeat addition by telomerase followed by semi-conservative DNA replication does not regenerate a G-overhang on the leading strand. Black arrowheads represent sequences added by telomerase. Wavy pink lines represent RNA primers. Parental DNA is indicated by black lines, and replicated DNA by orange lines. Adapted from Lingner et al., 1995.

Shelterin conceals the G-overhang from the DNA repair machinery. Shelterin is presumed to dictate telomere structure, which is thought to have implications in telomere replication and protection. One widely accepted model, proposed from mammalian studies, posits that shelterin protects telomeres from degradation by imposing a secondary DNA structure called the "t-loop" (Fig 1.5, (de Lange, 2005). In this model, the G-overhang folds back into the double-stranded DNA region of telomeres and invades it by pairing with the C-strand. In this way, the tips of telomeres would be hidden away from degradation. As a consequence, the G-strand at the double-stranded region is displaced, creating a structure known as a "D-loop".

This model is supported by several *in vitro* experiments. T-loops have been observed by electron microscopy of purified telomeric fragments of several model organisms (Cesare et al., 2003; Griffith et al., 1999; Murti and Prescott, 1999; Nikitina and Woodcock, 2004). They were also recreated *in vitro* using synthesized telomere oligonucleotides and purified Shelterin proteins from human cells and fission yeast (Stansel et al., 2001; Tomaska, 2004). In addition, a telomere looping structure has been suggested for budding yeast. This structure extends up to the subtelomeric region and is dependent on telomere and heterochromatin components (De Bruin et al., 2000; De Bruin et al., 2001; Poschke et al., 2012). However, it

remains to be confirmed that t-loops form *in vivo*, and if so, whether it is the predominant conformation of telomeres.

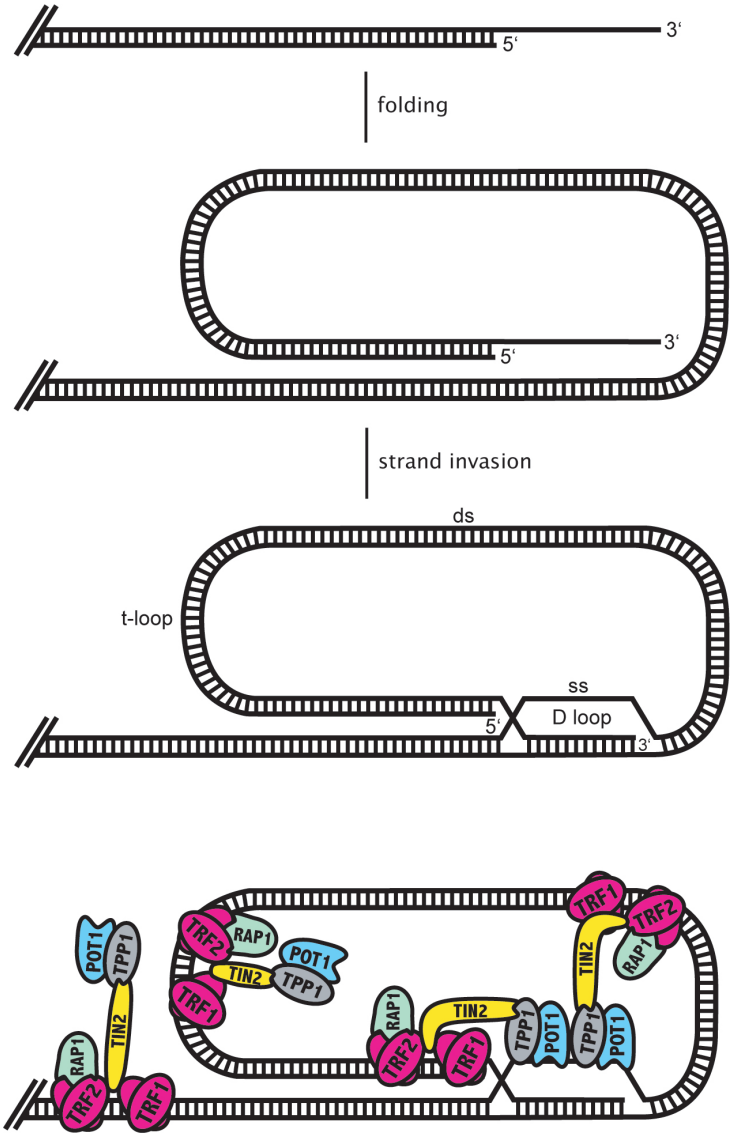


Figure 1.5. The t-loop model for telomere conformation and protection. The G-overhang folds back and invades the duplex telomeric DNA, generating a D-loop structure. The structure would be forced upon DNA by the interactions between single-stranded and double-stranded binding Shelterin components, and specifically by the DNA-bending properties of TRF1 and TRF2. Adapted from de Lange, 2005.

Although the single-stranded region and the double-stranded region of telomeres are bound directly by different shelterin components, they all associate into a single complex (Liu et al., 2004). The following sections explore the outcomes of disrupting these connections. For the sake of simplicity, the G-overhang binding Shelterin components are discussed in the following section, while the double-stranded components are discussed in section 1.4.2.

1.4.1 - G-overhang telomere components and their role in telomere protection and maintenance

Pot1 (Protection of telomeres) is a Shelterin component known to bind directly to the G-overhang (Figure 1.3; (Baumann and Cech, 2001; Liu et al., 2004; Miyoshi et al., 2008; Wang et al., 2007; Ye et al., 2004b). It was first discovered in *Oxytricha nova* as TEBP-alpha, which together with TEBP-beta form a single-stranded telomere binding complex (Gottschling and Zakian, 1986). Other orthologues are known in fission yeast, humans, chicken, mice, and plants (Baumann and Cech, 2001; Fang and Cech, 1991; Hockemeyer et al., 2006; Shakirov et al., 2010; Shakirov et al., 2005; Wei and Price, 2004). Pot1 possesses a high binding specificity to the G-strand of telomeres. In fission yeast, it protects telomeres from rampant degradation. Its absence leads to immediate and complete loss of telomere sequences, followed by chromosome-end fusions (Baumann and Cech, 2001; Bunch et al., 2005). As demonstrated by experiments using a conditional *pot1*$^{+}$ mutant, complete telomere degradation occurs in the ensuing cell cycle after Pot1 inactivation (Pitt and Cooper, 2010). Chromosome-end fusions resulting from Pot1 depletion are dependent on SSA components *rad22*$^{+}$ and *rad16*$^{+}$ (Figure 1.6; (Wang and Baumann, 2008). These fusions occur after considerable degradation (up to 13 Kb) of

chromosome ends and involve imperfect microhomologies in the subtelomeric regions. These microhomologies can range between 145-782 bp.

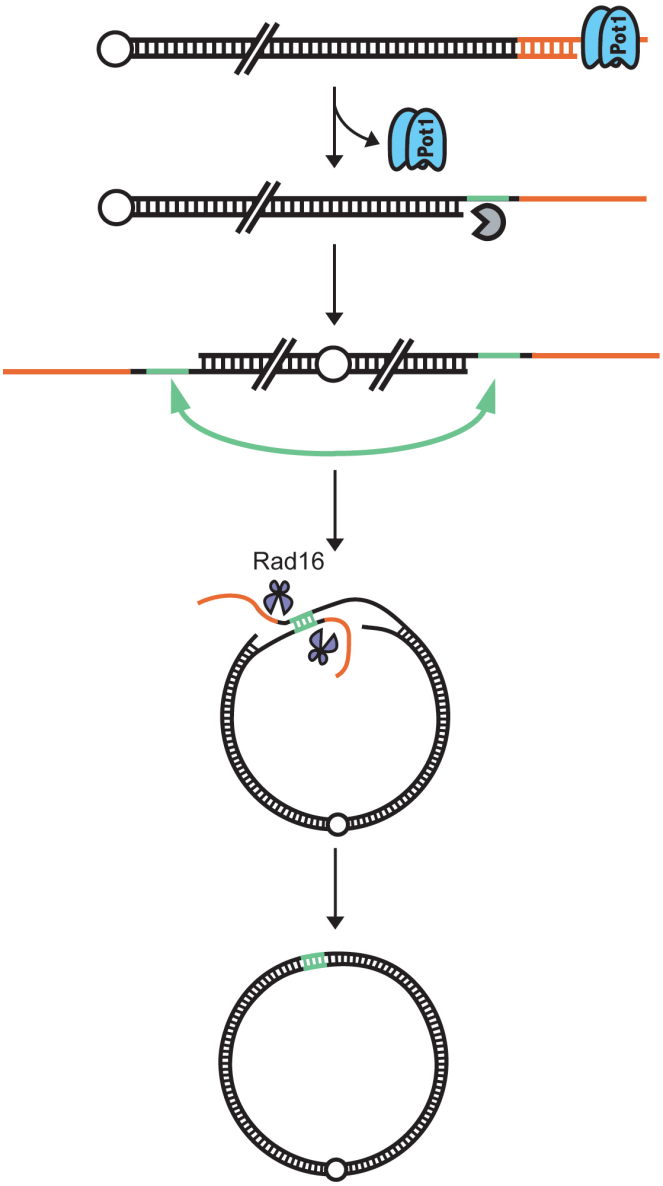


Figure 1.6. Complete resection of telomeres leads to circular survivors in fission yeast. Immediate degradation of telomeres, caused by the absence of Pot1, exposes regions of subtelomeric homology located 7-13Kb away from telomeric regions. Annealing of these homology regions generates intra-chromosome fusions through Rad16-dependent SSA. Orange strands represent telomere sequences. Microhomologies are represented in green. Exonucleases are represented in grey. Adapted from Dehé and Cooper, 2010.

In *Arabidopsis*, expression of a dominant-negative mutant of Pot1 orthologue AtPot2 also leads to telomere degradation and chromosome-end fusions (Shakirov et al., 2005). Accordingly, absence of functional Pot1 leads to activation of DNA damage checkpoints at telomeres in fission yeast, chicken DT40 cells and mice (Carneiro et al., 2010; Churikov and Price, 2008; Churikov et al., 2006; Denchi and de Lange, 2007; Guo et al., 2007; Hockemeyer et al., 2006). It is currently suspected that Pot1 competes with a DNA damage response (DDR) complex called RPA for recognition of the G-overhang (Barrientos et al., 2008; Flynn et al., 2011; Flynn et al., 2012). However, microscopy and ChIP experiments in fission yeast show co-localization of Pot1 and RPA (Carneiro et al., 2010). It has been suggested that Pot1 prevents activation of a DNA damage checkpoint by severing the downstream amplification of the DNA damage signal (Carneiro et al., 2010). Thus, Pot1 protects telomeres from being recognized as deleterious DNA double-strand breaks (DSBs). Chromosome-end fusions also occur in Pot1-deficient mice and chicken cells, albeit infrequently (Churikov et al., 2006; Hockemeyer et al., 2006). In mice, removing the mTPP1-POT1a/b complex results in microhomology-mediated fusions, in a NHEJ-independent, CtIP-dependent manner (Rai et al., 2010). In addition, absence of Pot1 in these higher eukaryotes results in G-overhang elongation, presumably from degradation of the 5'-strand. In mice there are two Pot1 orthologues with separate. mPOT1a inhibits DDRs at telomeres, while mPOT1b regulates G-overhang length. Therefore, it seems that telomere protection from degradation and evasion from DDR responses are separable Pot1 functions. Studies in human cells report that RNAi knockdown of human Pot1 (hPOT1) results in reduced cell viability and G-overhang shortening. This elicits a DDR that once again is not accompanied by extensive telomere degradation (Hockemeyer et al., 2005; Veldman et al., 2004; Yang et al., 2005). In agreement with these results,

removal of all shelterin components from mice telomeres does not result in evident telomere DNA degradation (Sfeir and de Lange, 2012). Thus, in comparison to mammalian models, fission yeast Pot1 seems to have an added role in preventing rampant DNA degradation. The role of Fission yeast's Pot1 in protecting telomeres from DNA repair may also be different from other organisms. Telomere degradation and fusions in fission yeast *pot1-1* cells are dependent on passage through the S phase (Pitt and Cooper, 2010). In contrast, chromosome-end fusions in double knockout mPOT1a/b-/- mice always involve both chromatids, suggesting that these fusions precede replication (Hockemeyer et al., 2006).

Pot1 also has additional functions as a telomere length modulator. Several fission yeast Pot1 mutants show telomere elongation phenotypes (Bunch et al., 2005; Pitt and Cooper, 2010). In addition, overexpression of hPOT1 promotes telomere elongation in a telomerase-dependent manner (Colgin et al., 2003). hPOT1 is thought to be a recruiter rather than an activator of telomerase, since increased concentrations of hPOT1 in cell lysates do not increase telomerase activity (Colgin et al., 2003). Moreover, *in vitro* telomere addition by telomerase is inhibited by increasing amounts of hPOT1 (Kelleher et al., 2005). Excess hPOT1 may compete with telomerase for telomere binding. However, this effect is not specific to hPot1 binding, as other single-stranded DNA binding proteins produce the same effect. hPOT1's telomerase recruitment function was illustrated by fusing hPOT1 with human telomerase catalytic subunit hTERT (Armbruster et al., 2004). This construct rescues the telomere shortening phenotype of a telomerase mutant in which the enzyme does not associate with telomeres. The mechanism of recruitment was further elucidated by removing the DNA-binding domain of hPOT1 (Loayza and de Lange, 2003). While remaining associated with telomeres through binding to other Shelterin components, this truncation results in a telomere elongation

phenotype. Thus, it may be that Pot1 is a telomerase recruiter, which also has negative telomere elongation functions while binding to the G-overhang.

In fission yeast, Pot1 recruits telomerase indirectly through another Shelterin component called Ccq1 (Figure 1.3). Ccq1 was first discovered to be in complex with the heterochromatin remodeling complex SHREC (Sugiyama et al., 2007). Later on, it was found to associate with Shelterin and recruit telomerase to telomeres (Khair et al., 2010; Miyoshi et al., 2008; Tomita and Cooper, 2008). Similarly to telomerase deletion, its absence results in gradual telomere erosion.

In addition, Ccq1 is also a telomere checkpoint inhibitor. *ccq1*Δ telomeres are subject to a Chk1-dependent DDR (Carneiro et al., 2010; Tomita and Cooper, 2008). Consequently, deleting *ccq1*$^+$ leads to *rad22*$^+$-independent, *rhp51*$^+$- and *rhp54*$^+$-dependent HR events at telomeres (Tomita and Cooper, 2008). As a result, liquid cultures of *ccq1*Δ mutants are able to maintain very short telomeres through a telomerase-independent pathway (Miyoshi et al., 2008; Tomita and Cooper, 2008).

Ccq1 binds to telomeres via Tpz1, which is a Shelterin component that is in complex with fission yeast Pot1 (Miyoshi et al., 2008). Known orthologues of Tpz1 include human and mouse TPP1, which are also partners of Pot1, and *Oxytricha nova*'s TEBP-beta, partner of TEBP-alfa (Gottschling and Zakian, 1986; Wang et al., 2007; Xin et al., 2007; Ye et al., 2004b). Tpz1 and its orthologues aid Pot1 binding to the G-overhang *in vivo* (Fang and Cech, 1993b; Gray et al., 1991; Kibe et al., 2010; Liu et al., 2004; Xin et al., 2007; Ye et al., 2004b). Concomitantly, absence of Tpz1 or of its orthologues recapitulates many phenotypes associated with Pot1 deficiency. In fission yeast, deleting Tpz1 results in massive chromosome-end degradation and fusions (Miyoshi et al., 2008). Absence of its mammalian counterpart, mTPP1, leads to checkpoint activation and mild

occurrence of chromosome-end fusions (Hockemeyer et al., 2007; Kibe et al., 2010; Xin et al., 2007). In mice, mTPP1 recruits both mPOT1a and mPOT1b to telomeres (Kibe et al., 2010). Together, mTPP1 and mPOT1a/b inhibit DDRs at telomeres through exclusion of RPA (Hockemeyer et al., 2006; Takai et al., 2011). Removing mTPP1 by itself results in the activation of a DDR and in checkpoint activation (Hockemeyer et al., 2007; Wu et al., 2006).

Knockout of mTPP1 also recapitulates the telomere length and G-overhang defects observed in the absence of mammalian mPOT1 (Xin et al., 2007; Ye et al., 2004b). In human cells, a specific domain of hTPP1 was found to be required for telomerase recruitment, independently of its telomere protection function (Nandakumar et al., 2012). *In vitro* and *in vivo*, hTPP1 binds directly to telomerase both in human and mice models (Abreu et al., 2010; Tejera et al., 2010; Xin et al., 2007), and in conjunction with hPOT1 increases telomerase processivity *in vitro* (Latrick and Cech, 2010).

In addition to the Pot1 complex, a second complex called CST binds to the G-overhang (Figure 1.3). It was first described in budding yeast, where it is composed of Cdc13, ScStn1 and ScTen1. Since then, it has been characterized in other organisms, including fission yeast and mammals (Gao et al., 2007; Gu et al., 2012; Martín et al., 2007; Miyake et al., 2009; Nakaoka et al., 2012; Song et al., 2008; Sun et al., 2009; Surovtseva et al., 2009; Wan et al., 2009). Only two of these subunits, Stn1 and Ten1, are known in *S. pombe* (Martín et al., 2007). In this organism, deletion of either *stn1*$^{+}$ or *ten1*$^{+}$ results in abrupt loss of telomere and subtelomere tracts, akin to *pot1*$^{+}$ deletion. In budding yeast, CST is the only known G-overhang binding complex (Figure 1.3). Its Cdc13 component has been considered to be a functional equivalent of Pot1 (Baumann and Cech, 2001). Concomitantly, absence of Cdc13 results in telomere degradation and checkpoint activation (Grandin and Charbonneau, 2007; Grandin et al.,

1997; Pennock et al., 2001; Vodenicharov and Wellinger, 2006). In addition, mutant alleles of *CDC13* have been shown to significantly increase the frequency of chromosome loss, accompanied by degradation of the 5'-strand up to subtelomeric regions (Garvik et al., 1995; Hartwell and Smith, 1985). This degradation occurs during G2/M, and not during G1 phase (Vodenicharov and Wellinger, 2006). This suggests that, as observed in fission yeast, telomere degradation requires passage through S phase.

Like Pot1, Cdc13 also regulates telomerase activity (Chandra et al., 2001; Grandin et al., 2000; Grandin et al., 1997; Lendvay et al., 1996; Pennock et al., 2001; Qi and Zakian, 2000). Cdc13 itself was shown to recruit components of telomerase (Evans and Lundblad, 1999; Nugent et al., 1996; Qi and Zakian, 2000).

Mutating either ScStn1 or ScTen1 produces similar phenotypes to Cdc13 dysfunction, including lethality, 5'-strand degradation, and telomere elongation (Grandin et al., 2001b; Grandin et al., 1997; Lin and Zakian, 1996; Nugent et al., 1996). In addition, overexpressing or artificially tethering ScTen1 or ScStn1 to telomeres can compensate Cdc13 dysfunction (Chandra et al., 2001; Grandin et al., 2001b; Pennock et al., 2001). This argues that Cdc13 protects telomeres partially through the components it recruits, as it is suggested for Pot1. However, Cdc13 also has independent functions. Telomere deprotection in a *CDC13-1* mutant is not compensated by artificially tethering ScStn1 and ScTen1 to telomeres (Grandin et al., 2001a). In addition, ScStn1 was proposed to inhibit telomerase recruitment by competing with Cdc13 for telomerase binding (Chandra et al., 2001; Grandin et al., 2000). A similar function was proposed for the human counterpart of CST. *In vitro* and *in vivo* ChIP studies show that CST competes with the hPOT1-hTPP1 complex for G-overhang binding (Chen et al., 2012b). CST has *in vitro* binding affinity for

telomeric G-rich oligonucleotides. It inhibits telomerase activity in two ways: by competing for G-overhang binding and by physically interacting with the hPOT1-hTPP1 complex (Chen et al., 2012b; Wan et al., 2009). In this way, it presumably interferes with telomerase recruitment or activation. In contrast to the hPOT1-hTPP1 complex, mammalian CST does not seem to be required for telomere protection, as absence of Cdc13 orthologue mCTC does not elicit checkpoint activation (Gu et al., 2012).

Thus, the G-overhang is populated by two protein complexes that may compete, but also interact with each other, possibly ensuring mutual regulation of their functions.

1.4.2 - Double-stranded telomere-binding components and their role in protection and telomere length regulation

The double-stranded region of telomeres interacts with a different set of proteins which, along with the Pot1 complex, complete the Shelterin complex. In fission yeast, its core component is Taz1, which binds directly to double-stranded telomere sequences via a Myb domain (Figure 1.3; (Cooper et al., 1997). The Myb domain forms a helix-turn-helix structure that is characteristic of transcription factors. This domain is also present in other telomere binding proteins including Taz1's human orthologues hTRF1 and hTRF2, and budding yeast's ScTbf1 and ScRap1 (Figure 1.3; (Bilaud et al., 1996; Broccoli et al., 1997; Chong et al., 1995; Konig et al., 1996; Liu and Tye, 1991). Several functions have been attributed to Taz1. Taz1 binding to telomeres results in a phenomenon known as telomere position effect, or TPE (Cooper et al., 1997). This phenomenon can be succinctly described as a reversible transcriptional silencing effect at telomere-adjacent genes (Gottschling et al., 1990). TPE has been observed in yeast and mammalian models (Baur, 2001; Gottschling et al., 1990; Levis et al.,

1985; Nimmo et al., 1994; Pedram et al., 2006). Furthermore, Taz1 is a key intervenient in telomere maintenance. It controls telomere length homeostasis by restraining telomerase recruitment (Cooper et al., 1997; Dehé et al., 2012; Miller et al., 2005). Its deletion results in telomere and G-overhang elongation, resulting in telomere sizes that range from 2.5 Kb to 4.5 Kb (Cooper et al., 1997; Tomita et al., 2003). This dramatic effect results from a deregulation of the timing of telomerase recruitment and its activity (Dehé et al., 2012). Normally, telomere elongation coincides with their replication, during late S phase (Kim and Huberman, 2001; Marcand et al., 2000; Moser et al., 2009a; Webb and Zakian, 2012). However, *taz1*Δ cells also extend telomeres outside of late S phase. Concomitantly, *taz1*Δ telomeres are accessible to telomerase during most of the cell cycle (Dehé et al., 2012). This contrasts with WT cells, in which telomerase only binds to telomeres during late S phase. Thus, Taz1 is involved in physically excluding telomerase from telomeres. This suggests that *taz1*Δ telomeres are overelongated because telomerase binds to telomeres throughout the cell cycle, and has a longer period of activity. This function of Taz1 seems to be evolutionarily conserved since, in mammalian cells, its orthologues mTRF1 and mTRF2 are also negative regulators of telomerase. Overexpressing or artificially tethering TRF1 and TRF2 to telomeres results in telomere shortening (Ancelin et al., 2002; Smogorzewska et al., 2000). Concomitantly, excluding hTRF1 from telomeres through the expression of a dominant-negative allele results in telomere elongation (Van Steensel and de Lange, 1997). hTRF1 seems to be part of a mechanism for telomere length regulation comprising several other components. Interactions between hPOT1 and hTRF1/2 have been suggested to play a role in telomere length homeostasis. Removing the DNA-binding domain of hPOT1 results in telomere elongation, even though hPOT1 remains at telomeres via interactions with Shelterin (Loayza and de Lange, 2003).

Telomere elongation in this mutant is abrogated by further removing the ability of hPOT1 to bind hTRF2 (Kendellen et al., 2009). Thus, it is possible that the hPOT1-TRF1/2 interaction is necessary to exclude telomerase activity. This interaction may cause structural alterations that are abolished when hPOT1 is unable to bind the G-overhang. The backbone of the hPOT1-hTRF1/2 interaction is another Shelterin component called hTIN2. hTIN2 binds to both hTRF1 and hTRF2 and mediates the association of hPOT1-hTPP1 to hTRF1 and hTRF2 (Figure 1.3; (Kim et al., 1999; O'Connor et al., 2006; Takai et al., 2011; Ye et al., 2004a; Ye et al., 2004b). TIN2's connection to both TRF components was shown to be important for Shelterin stability. Absence of TIN2 leads to a defect in telomere localization of the TRF proteins and of hPOT1-TPP1, thereby activating cell cycle checkpoints (Kim et al., 1999; O'Connor et al., 2006; Takai et al., 2011; Ye et al., 2004a; Ye et al., 2004b). Furthermore, removing TRF1 leads to a defect in TRF2 telomere localization in both mouse and human cells (Iwano et al., 2004; Ye et al., 2004a). Thus, TIN2 has a foundational role in the architecture of human Shelterin. Knockdown of TIN2 also leads to telomere shortening (Abreu et al., 2010). Although this result could be attributed to its function in recruiting TPP1, different TIN2 mutants are defective in telomerase recruitment without interfering with TPP1's telomere localization (Savage et al., 2008; Walne et al., 2008; Yang et al., 2011). In contrast, another TIN2 mutation, TIN2-13, causes telomere elongation even though it remains bound to TRF1 (Kim et al., 1999). This result suggests that TIN2 has an independent role in negative regulation of telomerase activity. However, it was not investigated whether the TIN2-13 mutation disrupts the TRF1-TRF2 association. This could destabilize TRF2 binding to telomeres, resulting in telomere elongation. In addition to TIN2, control of telomerase activity by TRF1 is also dependent on PINX1. PINX1 is recruited to telomeres via TRF1 and binds to telomerase, thereby inhibiting

its activity (Banik and Counter, 2004; Soohoo et al., 2011; Zhou and Lu, 2001). This inhibition is counter-balanced by Tankyrase, a protein that reduces TRF1 binding to telomeres (Smith and de Lange, 2000). Surprisingly, removing mTRF1 in the mouse model does not alter telomere length (Iwano et al., 2004).

In addition to their role in telomere length regulation, Taz1 and its orthologues share key roles in telomere maintenance. Absence of Taz1 results in a growth defect when cells are grown at 20ºC (Miller and Cooper, 2003). This defect may be caused by telomere entanglements that are left unresolved at lower temperatures, causing a G2/M checkpoint delay and chromosomal instability (Miller and Cooper, 2003; Miller et al., 2006). *taz1*$^{+}$ is also required for efficient progression of the replication fork. Its deletion causes replication fork stalling at telomeres (Miller et al., 2006). This phenotype may in fact be at the origin of the telomere entanglements observed in the *taz1*Δ mutant (Miller and Cooper, 2003; Miller et al., 2006). Similarly, hTRF1 prevents telomere fork stalling in human cells (Sfeir et al., 2009). In *taz1*Δ fission yeast cells, telomere fork stalling progresses into fork collapse in every round of replication (Dehé et al., 2012). Most of the telomeric tract is lost in the process. However, it is immediately re-synthesized by telomerase, allowing cells to progress normally throughout the cell cycle. In fact, *taz1*Δ telomeres are abruptly lost in the absence of telomerase (Miller et al., 2006). Replication fork stalling at *taz1*Δ telomeres may be caused by DNA structures called G-quadruplexes. These structures may form whenever guanine residues are allowed to bond with each other. At *taz1*Δ telomeres, they would occur during unwinding of the telomere structure, thus preventing progression of the DNA replication machinery (Miller et al., 2006). Similarly, TRF1 was suggested to prevent fork stalling by recruiting helicases that would unwind G-quadruplex structures (Sfeir et

al., 2009). Alternatively, Taz1 may be necessary to open telomeres for passage of the replication fork.

Replication fork stalling may cause *taz1*Δ telomeres to undergo recombination (Dehé et al., 2012). *taz1*Δ telomeres are subjected to *rad22*$^+$-dependent HR events during G_2 phase (Ferreira and Cooper, 2001, 2004; Nakamura et al., 1998). Accordingly, *taz1*Δ cells retain linear chromosomes and telomere sequences even after deletion of telomerase (Nakamura et al., 1998). Telomere maintenance in the double mutant requires the MRN complex and Tel1 (Subramanian et al., 2008).

Surprisingly, even though telomeres are subjected to repair, *taz1*Δ cycling cells show no decrease in viability or signs of chromosome fusions and instability. However, *taz1*Δ telomeres participate in chromosome-end fusions when cells are subjected to a G_1 arrest (Figure 1.7; (Ferreira and Cooper, 2001). In accordance to a predominance of NHEJ outside of G_2/M, these fusions do not occur in the absence of either *lig4*$^+$ or *ku70*$^+$. In fact, removing *rad22*$^+$ from *taz1*Δ mutants results in chromosome-end fusions, even in cycling cells (Ferreira and Cooper, 2004). Presumably, During G_2/M phases, *taz1*Δ mutants are subjected to upregulated HR at telomeres, which inhibits NHEJ. In contrast to chromosome-end fusions occurring in *pot1*Δ mutants, chromosome-end fusions in *taz1*Δ cells occur without loss of telomere signal.

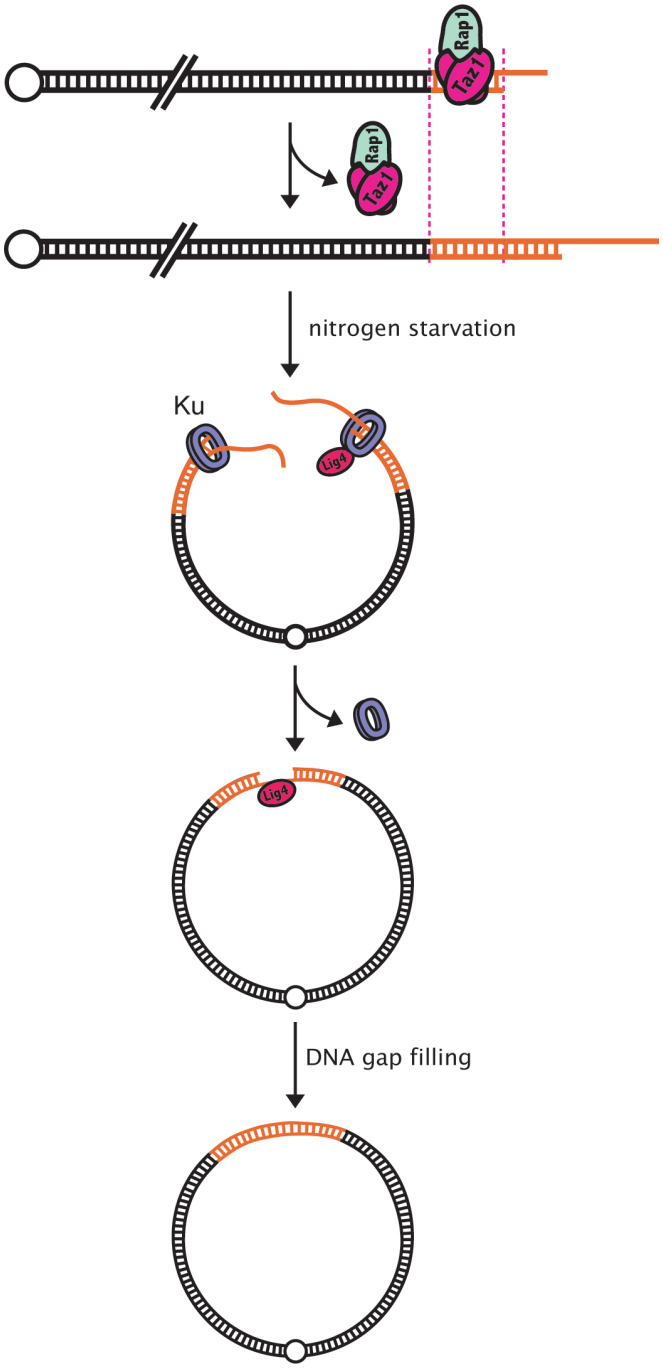


Figure 1.7. NHEJ-mediated telomere fusions occur with no loss of telomere sequences. Loss of Taz1 or Rap1 leads to Lig4 and Ku-dependent telomere fusions in G_1-arrested cells. Orange strands represent telomere sequences.

Similarly, expression of the dominant-negative mutant of human TRF2, TRF2ΔBΔM, results in DDR activation and NHEJ-mediated chromosome-end fusions during G_1 phase (Karlseder et al., 1999; Van Steensel et al., 1998). Loss of telomere signal is not visible in these fusions, as observed for *taz1*Δ mutants. A similar result is observed after deletion of mammalian TRF1 (Martínez et al., 2009). Concomitantly, simultaneous deletion of both TRF2 and TRF1 leads to chromosome fusions with no loss of telomeres (Bae and Baumann, 2007; Konishi and de Lange, 2008; Sfeir and de Lange, 2012; Yang et al., 2005).

Similar to mammalian systems, a second TRF homologue was recently discovered in fission yeast, called *tbf1*$^+$ (Pitt et al., 2008). This gene is essential and its product is able to bind *in vitro* to telomeric DNA. Tbf1 over-expression results in telomere elongation, suggesting that it plays a role in telomere length regulation.

Budding yeast also possesses a Taz1 orthologue called ScTbf1, which is only able to bind to subtelomeric regions (Berthiau et al., 2006; Brigati et al., 1993). While it doesn't retain the central role that Taz1 plays at fission yeast telomeres, ScTbf1 promotes telomerase activity specifically at short telomeres and is involved in inhibiting activation of DDRs at telomeres (Arnerić and Lingner, 2007; Berthiau et al., 2006; Fukunaga et al., 2012). Surprisingly, ScTbf1 is able to bind and maintain human-like TTAGGG telomere sequences engineered onto budding yeast chromosome ends (Brevet et al., 2003; Ribaud et al., 2011). It is speculated that during the evolutionary History of *S. cerevisiae*, its telomere consensus sequence was changed from the ancestral TTAGGG repeats to the TG1-3 sequence it currently holds. In the process, ScTbf1 would have lost the ability to bind to telomeres (Ribaud et al., 2011). Similarly, in fission yeast, a transcriptional regulator called Teb1 possesses two Myb domains and

binds specifically to the human telomere consensus sequence (Valente et al., 2013). However, Teb1 has no documented function at telomeres.

Some of fission yeast's Taz1 functions are also attributed to Rap1, a Shelterin component which is recruited to telomeres by Taz1 (Figure 1.3; (Kanoh and Ishikawa, 2001). Rap1 was first discovered in budding yeast (Berman et al., 1986; Shore and Nasmyth, 1987). It is an evolutionarily conserved Shelterin component, which also has known orthologues in mammals (Li et al., 2000). Deletion of fission yeast *rap1*$^{+}$ recapitulates several of *taz1*Δ phenotypes, including telomere and G-overhang elongation, G_1-specific chromosome-end fusions and disruption of TPE (Kanoh and Ishikawa, 2001; Miller et al., 2005). Artificially tethering Rap1 to *taz1*Δ telomeres does not compensate the G-overhang defects and chromosome-end fusions observed in the mutant (Miller et al., 2005). Thus, it is possible that Some Rap1 functions are Taz1-dependent. In fact, overexpressing Taz1 in *rap1*Δ cells partially suppresses its telomere elongation phenotype (Dehé et al., 2012). This suggests that some *rap1*Δ phenotypes are aggravated because Taz1 levels are insufficient to cover the whole extent of *rap1*Δ telomeres. In addition, unlike *taz1*Δ mutants, *rap1*Δ telomeres are not subjected to replication fork stalling and collapse (Miller et al., 2006). Thus, some telomere properties, such as chromosome-end protection and telomere length regulation, are most probably a collaborative process between Taz1 and Rap1.

Similar to fission yeast, human hRAP1 binds indirectly to telomeres via hTRF2 (Figure 1.3; (Li et al., 2000). Concomitantly, hRAP1 is a negative regulator of telomere length, which in conjunction with hTRF2 protects telomeres from chromosome-end fusions (Arat and Griffith, 2012; Bae and Baumann, 2007; Li et al., 2000; O'Connor et al., 2004). In fact, artificially tethering hRAP1 to telomeres prevents chromosome-end fusions independently of TRF2 (Sarthy et al., 2009). This suggests that hTRF2

prevents chromosome-end fusions solely by recruiting hRAP1. In mice, however, mRAP1 does not prevent chromosome-end fusions (Martínez et al., 2010). Despite this, mouse mRAP1 prevents recombination at telomeres (Sfeir et al., 2010).

rap1^{+} deletion may affect telomere elongation by disrupting Shelterin's structure. In fission yeast, this structure may be ensured by Poz1, which connects Tpz1 to Rap1 (Figure 1.3; (Fujita et al., 2012; Miyoshi et al., 2008). Accordingly, simultaneous deletion of *ccq1*$^{+}$ and *poz1*$^{+}$ results in immediate telomere loss and chromosome-ends fuse in a *pot1*Δ-like manner (Miyoshi et al., 2008). In fact, Poz1 seems to recapitulate the bridging function of human shelterin component hTIN2 (Figure 1.3, (Houghtaling et al., 2004; Liu et al., 2004; Takai et al., 2011; Ye et al., 2004a; Ye et al., 2004b). Like hTIN2, Poz1 is a negative regulator of telomere length, as deletion of *poz1*$^{+}$ results in telomere elongation (Miyoshi et al., 2008). Accordingly, removing the Poz1-interacting domain of Rap1 results in similar telomere length and TPE defects as in the Rap1 deletion mutant (Fujita et al., 2012). Therefore, Poz1's interaction with Rap1 should prevent Ccq1 from recruiting telomerase. In fact, recruitment of telomerase depends on Ccq1 phosphorylation by checkpoint kinases Tel1 and Rad3 (Moser et al., 2011; Yamazaki et al., 2012). Taz1, Rap1 and Poz1 inhibit this phosphorylation. Thus, Taz1, Rap1 and Poz1 may enforce a specific telomere configuration, which inhibits Ccq1 phosporylation and telomere elongation.

In budding yeast, ScRap1 binds directly to double-stranded telomere DNA (Buchman et al., 1988; Conrad et al., 1990; Lustig et al., 1990; Shore and Nasmyth, 1987; Wright et al., 1992). Like Taz1 in fission yeast, ScRap1 protects chromosome-ends from undergoing NHEJ-mediated telomere-to-telomere fusions (Pardo and Marcand, 2005). Additionally, ScRap1 is required for TPE and telomere length regulation

(Bonetti et al., 2010; Kyrion et al., 1992; Liu et al., 1994; Negrini et al., 2007; Sussel and Shore, 1991; Vodenicharov et al., 2010). Early experiments revealed that several *ScRAP1* mutations result in either telomere shortening or elongation (Kyrion et al., 1992; Liu et al., 1994; Lustig et al., 1990; Sussel and Shore, 1991). Accordingly, removing ScRap1 from telomeres results in telomere elongation (Lustig et al., 1990; Sussel and Shore, 1991). Furthermore, artificially tethering ScRap1 to telomere-proximal regions results in telomere shortening (Marcand et al., 1997). This shortening is proportional to the number of bound ScRap1 molecules. Thus, ScRap1 seems to function as a molecular counting mechanism of telomere length. This model lead to the idea that telomeres switch between two different conditions: a "closed" and an "open" state. In the "closed" state, telomeres are long and recruit ScRap1 molecules in sufficient number to repress telomere elongation. In the "open" state, which arises after telomeres erode, less ScRap1 molecules are recruited to telomeres. Consequently, telomerase is able to access telomeres. In the closed state, telomerase may be blocked by structural alterations promoted by ScRap1. In fact, *in vitro* experiments show that ScRap1 stimulates interactions between the G-overhang and the double-stranded region of telomeres, suggestive of a t-loop (Gilson et al., 1994). The telomere elongation observed for many other telomere mutants may therefore reflect the disruption of a higher order structure at telomeres, such as a t-loop. Figure 1.8 schematizes this model at fission yeast telomeres.

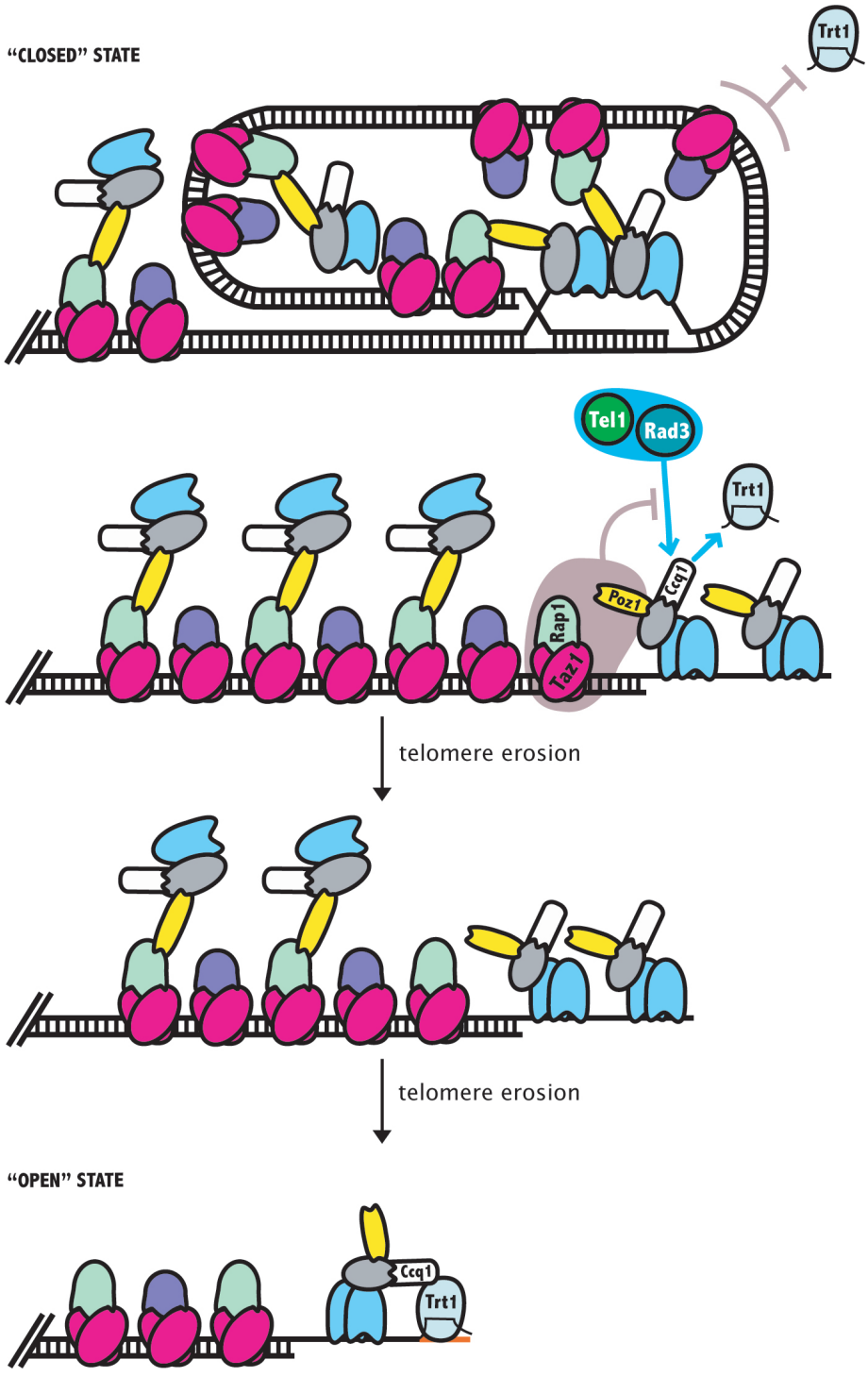

"CLOSED" STATE
Trt1
Tel1
Rad3
Trt1
Ccq1
Poz1
Rap1
Taz1
telomere erosion
telomere erosion
"OPEN" STATE
Ccq1
Trt1

Figure 1.8. Model for telomere length regulation in fission yeast. Telomeres may exist in two distinguishable configurations. In the "closed state", the G-overhang is concealed within the t-loop and is unavailable as substrate for telomerase. It is possibly in this conformation that Taz1, Rap1 and Poz1 inhibit phosphorylation of Ccq1 by Tel and Rad3, thus preventing telomerase recruitment. In the "open" state, telomeres become too short for a foldback structure to form, thus revealing the G-overhang to telomerase and allowing telomere elongation. Adapted from de Lange, 2005.

The counting mechanism model predicts that not all telomeres are equally elongated in a single cell cycle. In fact, in budding yeast the shortest telomeres are more likely to recruit telomerase and elongate during S phase (Bianchi and Shore, 2007b; Teixeira et al., 2004). By measuring single telomere elongation events in budding yeast, it was found that telomere repeat addition is a stochastic process whereupon not all telomeres are elongated (Teixeira et al., 2004). In this organism, the shortest telomeres have a higher probability of elongating. Additionally, telomere length does not influence the number of telomere repeats added during any single elongation event. However, these findings may not apply to all organisms. In human cancer cells, most telomeres are elongated at every cell cycle by a single telomerase molecule (Zhao et al., 2011). In this system, the number of added telomere repeats varies with telomerase availability, as overexpressing telomerase leads to an increase in the number of elongation events at a single telomere (Zhao et al., 2011). Therefore, what may be subject to regulation in human cells is the number of telomerase-mediated elongation events. This does not invalidate the conservation of a counting mechanism. In fact, overexpression of TRF1 or TRF2 leads to telomere shortening, suggesting a similar mechanism for telomere length control in humans (Smogorzewska et al., 2000; Van Steensel and de Lange, 1997).

In budding yeast, telomere elongation may be regulated by the timing of telomere replication. Accordingly, one study observes that shorter telomeres replicate earlier in the cell cycle than longer telomeres (Bianchi and Shore, 2007a). The authors propose that telomerase is able to access telomeres earlier, which induces firing of nearby origins of replication. Therefore, it is tempting to speculate that ScRap1 controls telomerase access to telomeres by determining the timing of telomere replication.

ScRap1 recruits two additional proteins to telomeres, the Rap1 interacting factors 1 (ScRif1) and 2 (ScRif2; (Hardy et al., 1992; Wotton and Shore, 1997). These components are not required for ScRap1-dependent chromosome-end protection (Marcand et al., 2008). However, deletion of either *ScRIF1* or *ScRIF2* results in TPE defects and modest telomere elongation. Furthermore, deleting both genes has a synergistic increase in telomere length, which is similar to the effect of ScRap1 removal from telomeres. In addition, artificially tethering ScRif1 and ScRif2 to telomeres abrogates the requirement of scRap1 for telomere length control (Levy and Blackburn, 2004). Thus, ScRif2 and ScRif2 are the key regulators of telomere length. Therefore, the telomere elongation phenotype observed with mutant versions of ScRap1 reflects their absence from telomeres (Liu et al., 1994; Wotton and Shore, 1997). Like ScRap1, ScRif2 promotes the formation of a fold-back structure at telomeres, further suggesting that telomerase access to telomeres is limited by this structure (Poschke et al., 2012). In addition, ScRif2 also protects chromosome-ends from undergoing NHEJ-mediated telomere-to-telomere fusions, in a ScRap1-dependent manner (Marcand et al., 2008).

In fission yeast, only a single Rif1 orthologue is known, which binds directly to Taz1 (Kanoh and Ishikawa, 2001). In this organism, Rif1 shows no association with Rap1. Instead, it has been proposed that Rif1 competes with Rap1 for Taz1 binding. Concomitantly, absence of Rap1 results in

increased association of Rif1 to telomeres (Kanoh and Ishikawa, 2001; Miller et al., 2005). Deleting *rif1*$^+$ results in only a modest increase (~200 bp) in telomere length (Kanoh and Ishikawa, 2001; Miller et al., 2005). Unlike *taz1*Δ or *rap1*Δ mutants, a G_1 arrest does not cause rif1Δ telomeres to become fusogenic. Furthermore, Rif1 is not required for TPE (Kanoh and Ishikawa, 2001). Deleting *rif1*$^+$ and *rap1*$^+$ simultaneously results in longer telomeres than in either mutant, suggesting that Rif1 and Rap1 regulate telomerase through different pathways.

The human orthologue of Rif1, hRIF1, is not a shelterin component, but a DDR factor that localizes to telomeres only when they become deprotected (Silverman, 2004). Along with checkpoint mediator 53BP1, hRIF1 inhibits 5'-resection (Di Virgilio et al., 2013; Zimmermann et al., 2013). This may be a conserved feature since budding yeast ScRif1 is similarly involved in inhibiting 5'-strand resection at telomeres (Anbalagan et al., 2011; Bonetti et al., 2010).

Thus, the mechanisms of telomere protection and length regulation seem to be broadly conserved in yeasts and mammals. Even though specific components involved in these processes may vary slightly, the evidence points towards common strategies of telomerase regulation and telomere protection.

1.4.3 - TERRA and transcription at telomeres

In addition to Shelterin, telomeres have an RNA component called TERRA. It is a non-coding RNA transcribed from subtelomeric and telomeric regions, which in mammalian models associates with TRF1/2 (Azzalin et al., 2007; Bah et al., 2012; Deng et al., 2009b; Greenwood and Cooper, 2012; Luke et al., 2008; Schoeftner and Blasco, 2007). Disruption of Shelterin specific components, which establish TPE, results in increased

expression of these transcripts. This has been observed in budding and fission yeast's Rap1 and Rif mutants (Greenwood and Cooper, 2012; Iglesias et al., 2011). TRF2 mutants in human cells also show increased TERRA levels (Caslini et al., 2009). Several experiments suggest that TERRA inhibits telomerase. In budding yeast, inhibiting TERRA degradation results in its accumulation, which correlates with telomere shortening (Luke et al., 2008). In human cells, TERRA upregulation is accompanied by a complete loss of the telomere tract (Azzalin et al., 2007). *In vitro* experiments using TERRA-like oligonucleotides were shown to inhibit telomerase activity (Schoeftner and Blasco, 2007). Accordingly, forcing transcription at budding yeast telomeres leads to telomere shortening in *cis* (Sandell et al., 1994). However, it is not yet clear how TERRA transcription affects telomere length. It is possible that the accumulation of TERRA prevents telomerase recruitment or activation. Alternatively, increased passage of the transcription machinery through telomeres could inhibit telomerase recruitment or activation. Contrary to these results, recent experiments in human cells reveal no correlation between increased transcription of TERRA and telomerase inhibition (Farnung et al., 2012). It is possible that, depending on the strength of the promoter, forcible transcription at telomeres disrupts telomere structure. Thus, the role of TERRA in telomere length regulation remains somewhat controversial.

1.4.4 - Telomerase and mechanisms of telomere maintenance

Critical telomere shortening frequently results in chromosome-end fusions (Capper et al., 2007; Nakamura et al., 1998). In the majority of organisms, chromosome-end fusions lead to genomic instability and death (Blasco et al., 1997; Karlseder et al., 1999; Lundblad and Szostak, 1989).

To prevent genomic catastrophe, the large majority of eukaryotic organisms uses telomerase to replenish the ends of chromosomes with new telomere repeats (Kim et al., 1994; M Zahler and M Prescott, 1988; Mantell and Greider, 1994; Morin, 1989; Nakamura et al., 1997; Prowse et al., 1993; Shippen-Lentz and Blackburn, 1989; Yu and Blackburn, 1991; Yu et al., 1990). Absence of any of telomerase's components leads to gradual telomere shortening (Beernink et al., 2003; Blasco et al., 1997; Counter et al., 1997; Lendvay et al., 1996; Leonardi et al., 2008; Lundblad and Blackburn, 1993; Nakamura et al., 1997; Nakamura et al., 1998; Singer and Gottschling, 1994).

In fission yeast, there are three known components of telomerase: a catalytic subunit called Trt1, the RNA template component TER1, and an accessory factor called Est1. The catalytic subunit of telomerase adds new telomere repeats in tandem by using the RNA component TER1 as a template (Cohn and Blackburn, 1995; Counter et al., 1997; Leonardi et al., 2008). *In vivo* telomerase activity requires Est1, which along with TER1 is necessary to deliver telomerase to telomeres (Beernink et al., 2003; Webb and Zakian, 2012). The first Est1 orthologue was discovered in budding yeast (Lundblad and Szostak, 1989). In this organism, ScEst1 functions simultaneously as a recruiter and activator of telomerase by binding to Cdc13, the RNA template component TLC1 and the telomerase catalytic subunit Est2 (Evans and Lundblad, 1999; Evans and Lundblad, 2002; Qi and Zakian, 2000; Taggart et al., 2002; Virta-Pearlman et al., 1996; Zhou et al., 2000). A similar mechanism of recruitment is found in fission yeast (Webb and Zakian, 2012). In this model, Est1 binds to telomeres during late S phase, in a Ccq1-, Trt1- and TER1-dependent manner.

Like most organisms, fission yeast loses viability upon telomere erosion (Nakamura et al., 1998). However, some fission yeast telomerase mutant cells are able to survive these fusions. Gradual erosion of telomeres

in *trt1*Δ mutants leads to chromosome-end fusions (Nakamura et al., 1998). Fission yeast only possesses three chromosomes – fairly few when compared with budding yeast (16) or humans (23). As a consequence, intrachromosomal fusions are frequent, generating functional circular chromosomes that maintain the integrity of the genome (Nakamura et al., 1998). Chromosome circularization in *trt1*Δ mutants is reported to be *rad16*$^{+}$-dependent (Wang and Baumann, 2008). Therefore, circularization of *trt1*Δ chromosomes seems to be SSA-dependent, as was observed for *pot1*Δ mutants (Wang and Baumann, 2008). Accordingly, Southern blot analysis of circular chromosomes from *trt1*Δ survivors showed at least 5 Kbp resection of chromosome ends prior to fusing (Nakamura et al., 1998).

In budding yeast *est1* mutants, microhomology-mediated chromosome-end fusions have also been reported (Hackett et al., 2001). Of 12 chromosome-end fusions analyzed by PCR, all showed complete resection of telomeres. Furthermore, 7 of these fusions possessed 1-10 bp microhomologies at fusion junctions. These fusions do not require ScLig4. NHEJ-mediated chromosome-end fusions were also reported in budding yeast Type II survivors (Liti and Louis, 2003). Nevertheless, it is unknown whether these fusions retain telomere sequences at the fusion junctions.

Interestingly, chromosome-end fusions from telomerase mutants of Arabidopsis have more diverse outcomes (Heacock et al., 2004). These can be telomere-to-telomere, telomere-to-subtelomere, or subtelomere-to-subtelomere, with some fusion junctions containing DNA insertions of unknown origin. Microhomologies of up to 12 bp could be found at fusion junctions. When visible, DNA resection ranged from 44 to 459bp from the beginning of the telomeric tract. Deleting At*ku70* along with telomerase resulted in a much higher percentage of microhomologies (83%), than for the single telomerase mutants (39%), and no DNA insertions were found. This suggests that AtKu inhibits the microhomology-mediated pathway of

repair at dysfunctional telomeres. Surprisingly, fusions seem to be independent of AtLig4, although absence of AtLig4 in the triple mutant At*tert* At*ku70* At*lig4* causes increased telomere erosion. In this case, an average of 2 bp microhomologies is still prevalent at the fusion junctions, as opposed to the 4.6 bp average of microhomologies observed in the *tert ku70* double mutants (Heacock et al., 2004). In conformity with budding and fission yeast's MMEJ, chromosome-end fusions in Arabidopsis are dependent of MRN component AtMre11.

In the case of *C. elegans* telomerase mutants, most chromosome-end fusions are either subtelomere-to-subtelomere or telomere-to-subtelomere type, with a minority (3%) showing telomeres from both sides of the fusion (Lowden et al., 2008). Chromosome-end fusions from telomerase mutants have no homologies at the fusion junctions. However, microhomologies were evident after removing NHEJ by deleting *lig-4* (Lowden et al., 2008), once again showing that microhomology-mediated repair is inhibited by NHEJ.

In homozygous mutant mice for the telomerase RNA component mTR, analysis of 8 chromosome-end fusion junctions revealed microhomology mediated fusions at satellite DNA upstream of telomere sequences (Hemann et al., 2001).

In humans, chromosome-end fusions were studied by artificially bypassing senescence in a variety of cell culture models. These included fibroblasts, immortalized cell lines that constitutively express telomerase, and cancer cell lines (Capper et al., 2007). In these cases, the emergent fusions had either critically short telomere-to-subtelomere, or subtelomere-to-subtelomere junctions. Almost 90% of these fusions were microhomology-mediated, supporting a role for SSA-like mechanisms of repair in this system as well.

Thus, it seems that, in contrast to the NHEJ-dependent telomere-to-telomere fusions, eroded chromosome ends generally fuse through microhomology-mediated mechanisms. Opposing telomere ends are inverted tandem repeats and are thus less likely to harbor complementary sequences at each side. Therefore, telomeres may passively inhibit microhomology-mediated end-joining mechanisms because they lack the necessary complementarity. As eroded chromosome ends lose their telomeres, they may become substrate to microhomology-mediated fusions as they are more likely to exhibit microhomologies.

Chromosome circularization is the preferred method of survival for *trt1*Δ fission yeast cells growing in solid media (Nakamura et al., 1998). However, if grown in liquid media, some survivors arise from telomeric crisis with linear chromosomes (Nakamura et al., 1998). These survivors show amplification of subtelomeric sequences suggestive of an HR-dependent mechanism. Thus, it is presumed that linear *trt1*Δ survivors emerge through HR-dependent telomere maintenance (Figure 1.9).

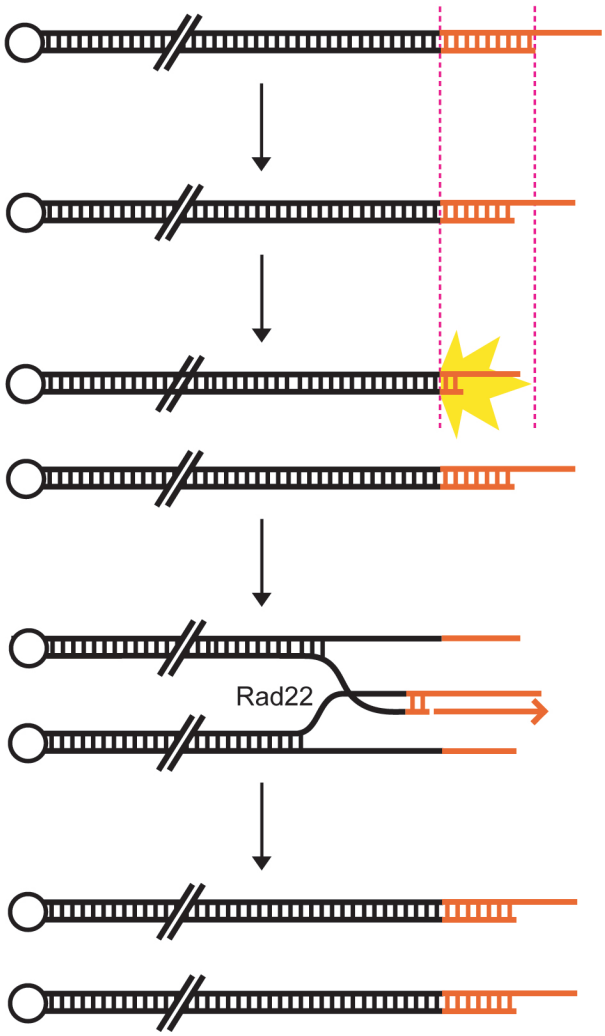


Figure 1.9. Linear survivors of telomere dysfunction. Loss of telomerase components leads to gradual telomere shortening until chromosome-end protection becomes compromised. At this point, a DDR response is triggered (represented by a yellow flash). Critically short telomeres may be elongated by Rad22-dependent HR. Orange strands represent telomere sequences. Adapted from Dehé and Cooper, 2010.

In these survivors, chromosome-end fusions are averted by maintaining short telomeres. However, a rarer type of linear survivors are able to maintain linear chromosomes in the absence of telomeres (Jain et al., 2010). These are called HAATI survivors. These survivors maintain linear chromosomes by replacing telomeres with amplified terminal rDNA or subtelomeric sequences (Figure 1.10; (Jain et al., 2010). Amplification of these sequences is dependent on the Rhp51 recombinase. It is thought

that these highly heterochromatic regions protect chromosome ends by recruiting Pot1 via an interaction with SHREC-Ccq1.

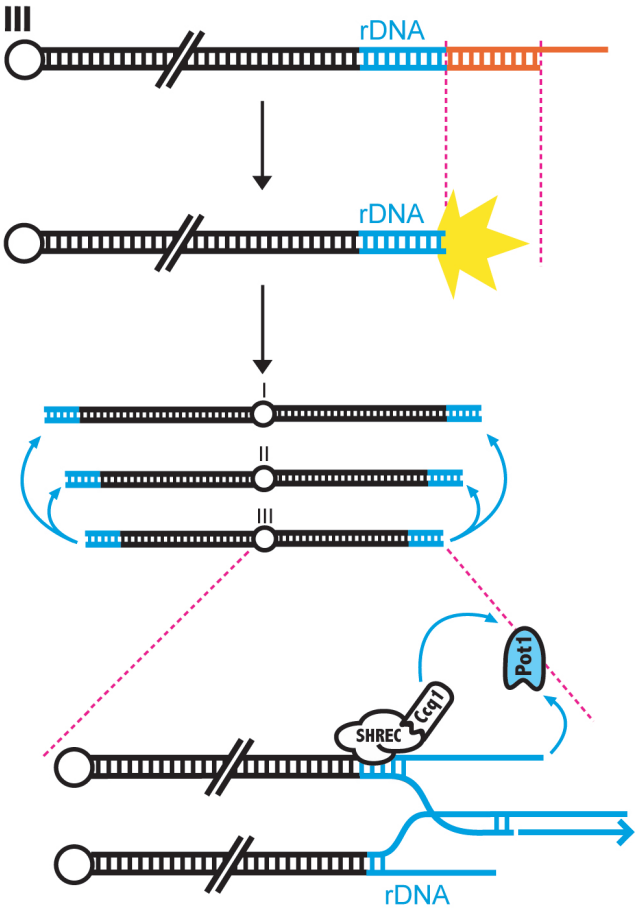


Figure 1.10. HAATI survivors. After gradual telomere erosion and complete loss of the telomere tract, cells may resort to another mode of survival, in which heterochromatic sequences, such as rDNA, are amplified at chromosomed ends. It has been suggested that these heterochromatin regions recruit Ccq1 via SHREC. Pot is thought to be anchored to these heterochromatic regions by interacting with Ccq1, thus maintaining chromosome linearity. Orange strands represent telomere sequences. The yellow flash represents a DDR response. Adapted from Dehé and Cooper, 2010.

Budding yeast is also able to survive in the absence of functional telomerase through HR-based mechanisms of telomere maintenance (Lundblad and Blackburn, 1993). There are two described types of survivors for this organism. Type I survivors amplify specific subtelomeric regions, while keeping very short telomeric repeats. This type of survivor is not very stable and converts to Type II survivors (Teng et al., 2000). These maintain the integrity of chromosome ends by recombining telomere tracts, which become very long and heterogenous in size (Teng and Zakian, 1999). Type I and Type II survivors distinct recombinatorial pathways (Lundblad and Blackburn, 1993) (Teng and Zakian, 1999). Both depend on ScRad52-dependent recombination. However, Type I survivors additionally require the ScRad51 recombinase, while Type II survivors are dependent of ScRad50 (Teng et al., 2000). Experiments in the yeast *Kluyveromyces lactis* suggest a model for HR-dependent telomere maintenance (Groff-Vindman et al., 2005; Natarajan et al., 2003; Natarajan and McEachern, 2002; Topcu et al., 2005; Xu and McEachern, 2012a; Xu and McEachern, 2012b). The amplified telomere tracts are homogenous, suggesting that recombination always involves the same donor source. The authors propose a "roll and spread" model, where a single short telomere is elongated by using a telomeric DNA circle as a template. This elongated telomere is then capable of recombining with all other telomeres, providing a sufficiently long telomere template for stable maintenance of chromosome ends. Accordingly, telomeric DNA circles have also been associated with recombination-based telomere maintenance in *S. cerevisiae* (Lin et al., 2005).

In addition, budding yeast can use another alternative pathway to maintain genomic integrity in a telomerase-independent, recombination-independent manner (Maringele and Lydall, 2004). In the absence of the

Exo1 nuclease, chromosome ends may survive complete telomere erosion by synthesizing large palindromes from pre-existing inverted repeats.

Human telomeres can also overcome the absence of telomerase (hTERT) through a recombination mechanism known as ALT (Alternative Lengthening of Telomeres; (Bryan et al., 1997). ALT has been calculated to be present at approximately 10% of all cancers, which do not show telomerase-dependent telomere elongation (Shay et al., 2012). ALT mechanisms may indeed pose an additional challenge to cancer therapies based on telomerase inhibition. Accordingly, experiments using conditional inhibition of telomerase showed that cancer cells are able to adapt and resort to ALT mechanisms of telomere maintenance, thus ensuring their continued proliferation (Hu et al., 2012). It remains to be seen whether the HR-based telomere maintenance mechanisms observed in yeast models are applicable to ALT mechanisms in cancer cells.

This section elaborated on the mechanisms with which telomeres guarantee chromosomal integrity and the perpetuation of the eukaryotic genome. Moreover, it described several consequences for telomere dysfunction. Telomeres have the three-fold responsibility of 1) ensuring complete replication of chromosome ends, 2) preventing chromosomes from being recognized as DNA breaks and 3) protecting chromosome ends from the DNA repair machinery. However, as will be discussed in the next section, Shelterin composition varies within the cell cycle. Furthermore, additional players are required for maintenance of the telomere structure, in particular components of the DDR machinery. This apparent contradiction is at the heart of chromosome-end protection and underlines the dynamic nature of telomeres.

1.4.5 - Telomeres are dynamic structures that employ DDR components in a cell cycle-dependent manner

Telomeres are privileged DNA DSBs for two reasons: 1) they do not trigger cell cycle checkpoints and 2) they are normally immune to DNA repair. One would expect that telomeres achieve these properties by actively blocking engagement of DDR components at chromosome ends. However, several cell cycle checkpoint and DNA repair components are required for telomere protection and maintenance (Longhese et al., 2000; Matsuura et al., 1999; Nugent et al., 1998; Ritchie et al., 1999; Ritchie and Petes, 2000; Tsukamoto et al., 2001). Some are transiently recruited to telomeres, particularly during late S/G2 phases, coinciding with the timing of telomere replication and elongation by telomerase in yeasts (Diede and Gottschling, 1999; Ferguson et al., 1991; Marcand et al., 2000; Moser et al., 2011; Moser et al., 2009a; Nakamura et al., 2002; Verdun et al., 2005; Verdun and Karlseder, 2006; Wellinger et al., 1993b; Wright et al., 1999; Yamashita et al., 1997; Zhu et al., 2000).

Similarly, the localization of Shelterin components at telomeres is cell cycle-dependent. Changes in their association with telomeres coincide, in some cases, with transient modifications of the telomere tract, such as loss and generation of the G-overhang (de Lange, 2002; Moser et al., 2009a; Taggart et al., 2002; Wellinger et al., 1993b). Thus, telomeres are constantly remodeled throughout the cell cycle (Verdun et al., 2005; Verdun and Karlseder, 2006).

In fission yeast, ChIP experiments on Shelterin and DDR components demonstrated the extent of telomere remodeling (Moser et al., 2009a). Trt1, Pot1 and Stn1 are recruited to telomeres during late S phase, in agreement with Pot1's indirect role in telomerase recruitment. Taz1 is present at telomeres throughout the cell cycle, with a two-fold binding decrease during late S phase. This is somewhat surprising, given Taz1's

role in aiding telomere DNA replication (Miller et al., 2006). Rap1 binding, however, showed no significant alterations throughout the cell cycle. Even though ChIP experiments were unable to find Rap1 in *taz1*Δ telomeres, it may remain residually at telomeres independently of Taz1, possibly by binding to Poz1 (Miller et al., 2005; Subramanian et al., 2008).

In contrast, budding yeast's ScRap1 peaks telomere binding during G_2 phase, while showing minimal binding (two-fold decrease) throughout S phase (Smith et al., 2003). However, ScRap1 binds independently to telomeres and its binding dynamics may differ from fission yeast's Rap1. In fact, ScRap1 telomere binding profile resembles that of Taz1, suggesting that both are evicted from telomeres by the same mechanisms. Surprisingly, even though ScRif1 and ScRif2 associate with telomeres through ScRap1, their telomere binding profile does not directly reflect this dependence (Smith et al., 2003). While ScRif1 shows two peaks of telomere binding, one through early G_1 and another during early S phase, scRif2 shows minimal binding to telomeres during G_2 phase, and peak binding during G_1/S.

Budding yeast's telomerase auxiliary factor ScEst1 was shown to bind to telomeres exclusively during S phase (Taggart et al., 2002). Somewhat unexpectedly, telomerase's catalytic subunit Est2 binds to telomeres throughout the cell cycle, with clear peaks during G1 and late S phase (Taggart et al., 2002). Mutations in different *est* genes affected Est2's telomere binding differently, reducing only either one of the two binding peaks (Chan et al., 2008). These results suggest that, at least in budding yeast, there are two independent Est2 recruitment mechanisms.

In human cells, telomere replication occurs throughout S phase (Ten Hagen et al., 1990; Wright et al., 1999). In accordance, recruitment of the catalytic and RNA subunits of telomerase occurs throughout S phase (Jády et al., 2006; Tomlinson et al., 2006). ChIP experiments were

conducted in primary fibroblasts to analyze the cell cycle-dependent variations in telomere binding of several Shelterin components (Verdun et al., 2005). These experiments show that TRF1 lowers its binding during S phase and then during G_2. TRF2 and hPOT1 also decrease their binding during G_2, and hPOT1 is present throughout S phase, according with its role in telomerase recruitment. Coinciding with hPOT1's decrease at telomeres, the TPP1-hPOT1 telomere binding competitor hCST associates with telomeres more prominently during late S phase and early G_2 (Chen et al., 2012b).

Thus, in yeasts and humans, there are periods during which Shelterin is normally incomplete, or at least some of its components are less represented. It is not inconceivable, then, that telomeres may become accessible to opportunist DDRs. In fact, telomeres are transiently recognized as DNA damage. Accordingly, binding of key DDR components to telomeres has been reported (Moser et al., 2009a; Verdun et al., 2005). RPA, the ssDNA-binding DDR initiator, binds to telomeres in late S phase both in fission and budding yeasts (Moser et al., 2009a; Ono, 2003; Schramke et al., 2003). A study using human cell extracts proposes that RPA competes with the hPOT1 complex for binding at the G-overhang (Flynn et al., 2011). Even though hPOT1 is unable to displace RPA from naked single-stranded telomeric DNA, RPA seems to be outcompeted by another ssDNA-binding protein called hnRNPA1. The authors' model proposes that there is a cyclical turnover between RPA, hnRNPA1, and hPOT1, regulated by cellular levels of TERRA. High levels of TERRA during early S phase bind to and sequester hnRNPA1 away from telomeres. As concentration of TERRA declines, hnRNPA1 displaces RPA away from telomeres during late S phase. After S phase, TERRA levels increase once again, allowing hPOT1 to take the place of hnRNPA1 at the telomeric 3'-overhang. Supporting this model is the find that siRNA-

mediated TERRA inhibition results in the activation of a DRR at human telomeres (Deng et al., 2009b).

For budding and fission yeasts, the role of telomere-bound RPA is to promote telomerase recruitment (Luciano et al., 2012). Concomitant with its role in generating the G-overhang, Mre11 is required for RPA recruitment to the leading strand telomere, but not the lagging strand telomere. In budding yeast, RPA seems to achieve this by recruiting ScEst1 to telomeres during S phase, in a ScKu70-dependent manner (Schramke et al., 2003). In fission yeast, RPA interacts specifically with telomerase's RNA component TER1. In agreement with these results, a mutant allele of one of budding yeast's RPA components, *rfa1-D228Y*, leads to shorter telomeres when in conjunction with deletion of ScKU70 (Smith et al., 2000). An analogous mutation in fission yeast, *rad11-D223Y*, produces short telomeres by itself, while still binding to telomeres (Ono, 2003). Deleting *taz1*$^{+}$ from this mutant results in immediate telomere resection, as observed for *taz1*Δ *trt1*Δ double mutants or *pot1*Δ mutants (Kibe et al., 2007). Deletion of the DNA helicase gene *rqh1*$^{+}$ rescues the chromosome-end resection phenotypes of both the *taz1*Δ *rad11-D223Y* double mutant and of *pot1*$^{+}$ deletion (Takahashi et al., 2011). This rescue is provided by Rhp51-dependent telomere maintenance, at least for *pot1*Δ *rqh1*Δ mutants. Nevertheless, it remains to be determined whether the phenotype observed for the *taz1*Δ *rad11-D223Y* double mutant results from defective telomerase recruitment caused by the *rad11-D223Y* allele. Alternatively, in the absence of RPA, *taz1*Δ telomeres are sensitive to resection prompted by the activity of Rqh1, as observed for *pot1*Δ *rqh1*Δ cells.

The Ku complex is another DNA repair component which has been implicated in telomere maintenance. It binds to telomeres and inhibits HR at subtelomeric regions, similarly to what is observed at DNA DSBs (Baumann and Cech, 2000; Bianchi and de Lange, 1999; d'Adda di

Fagagna et al., 2001; Gravel et al., 1998; Hsu et al., 1999; Kibe et al., 2003; Langerak et al., 2011; Miyoshi et al., 2003; Nakamura et al., 2002; Subramanian et al., 2008). In fission and budding yeasts, absence of any of Ku's two subunits results in short but stable telomere ends (Baumann and Cech, 2000; Boulton and Jackson, 1996a, 1998; Nugent et al., 1998; Porter et al., 1996). This effect does not seem to be epistatic with telomerase defects. Removing Ku from *trt1*Δ cells accelerates resection and loss of telomere sequences, suggesting that absence of Ku exposes telomeres to degradation (Baumann and Cech, 2000). Accordingly, fission and budding yeast Ku70 protect telomeres from MRX- and Exo1-mediated degradation during G_1 phase. Concomitantly, its absence results in abnormally long G-overhangs (Bonetti et al., 2010; Gravel et al., 1998; Kibe et al., 2003; Vodenicharov et al., 2010). In addition, fission yeast Ku protects chromosome-ends from Rhp51-mediated recombination (Baumann and Cech, 2000; Kibe et al., 2003). In budding yeast, but not in fission yeast, absence of ScKu results in loss of TPE (Gravel et al., 1998; Manolis et al., 2001). In fact, in budding yeast, telomeres act as a reservoir for ScKu, in the absence of a DDR (Martin et al., 1999).

In mammalian cells, absence of Ku also results in short telomeres (d'Adda di Fagagna et al., 2001). In this case, the phenotype is accompanied by an increase in chromosome-end fusions and genomic instability. Thus, Ku has a conserved role in telomere protection.

In addition to its role in telomere protection, Ku is involved in telomerase recruitment. For budding yeast, ScKu seems to promote Cdc13-dependent recruitment of telomerase (Grandin et al., 2000). It binds to TLC1 and is involved in the accumulation of telomerase components at telomeres. These include Est2 and ScEst1, which accumulate at G_1 and S phase, respectively (Chan et al., 2008; Fisher et al., 2004; Peterson et al., 2001; Stellwagen et al., 2003). Nevertheless, *in vitro* experiments reveal

that ScKu binds to telomere DNA or to TLC1 in a mutually exclusive manner (Nandakumar and Cech, 2013; Pfingsten et al., 2012). Thus, it is improbable that ScKu tethers telomerase directly to telomeres.

Many other DDR components are involved in telomere maintenance. Checkpoint kinases Tel1 and Rad3 emerged as two important, albeit redundant, components for telomere maintenance in fission yeast (Matsuura et al., 1999; Naito et al., 1998). While absence of either leads to short but stable telomeres, the double mutant circularizes chromosomes after complete telomere erosion. This suggests that Tel1 and Rad3 are independently involved in telomere maintenance. This function seems to be broadly conserved in other organisms. Disruption of budding yeast orthologues ScTel1 and Mec1, as well as mammalian ATM results in a similar telomere shortening phenotype and chromosome-end fusions (Lustig and Petes, 1986; Metcalfe et al., 1996; Ritchie et al., 1999; Vaziri et al., 1997; Xu and Baltimore, 1996). Budding yeast *Sctel1 mec1* double mutants are subjected to ScLig4-dependent telomere-to-telomere and telomere-to-subtelomere fusions (Mieczkowski et al., 2003). Similarly, an increase in NHEJ-mediated fusions between an induced DSB and short telomeres was reported for *Sctel1 mec1* and *Sctel1 tlc1* double mutants (Chan and Blackburn, 2003). Thus, removing checkpoint kinases Tel1 and Rad3/Mec1 compromise telomere length homeostasis and protection.

Epistasis analysis in fission yeast uncovered additional components of the Tel1 and Rad3 pathways of telomere maintenance (Dahlén et al., 1998; Matsuura et al., 1999; Nakamura et al., 2002). Three epistasis groups were identified in the Rad3 pathway, including Rad3/Rad26, Rad1/Rad9/Hus1/Rad17 and Ku70; and one group in the Tel1 pathway, including Rad32. Orthologues of these DDR components play a role in telomere maintenance in budding yeast, including MRX, which also shares an epistatic relationship with scTel1 (Longhese et al., 2000; Ritchie et al.,

1999; Ritchie and Petes, 2000; Tsukamoto et al., 2001). Consistent with their role in telomere maintenance, ChIP experiments confirmed the presence of Rad3, Rad26, Rad1, Rad9, Hus1 and Rad17 at fission yeast telomeres. Rad26 is usually recruited to ssDNA by RPA (Zou and Elledge, 2003). It is recruited to telomeres during late S phase, coinciding with RPA telomere binding (Moser et al., 2009a). Budding yeast ScTel1 is found at short telomeres mostly throughout S and G_2 phases (Sabourin et al., 2007). Similarly, its human orthologue ATM binds to telomeres late in S phase but particularly during G_2, coinciding with decreased hPOT1 binding (Verdun et al., 2005).

Tel1 and Rad3 are in fact involved in telomerase recruitment. Both are redundantly required for phosphorylation of the telomerase recruiter Ccq1 (Moser et al., 2011; Moser et al., 2009b). Furthermore, in the absence of both, Ccq1 is unable to recruit telomerase. This phosphorylation step is necessary for the interaction of Ccq1 and the Est1 subunit of telomerase. In fact, budding yeast ScTel1's kinase activity is also required for telomere elongation (Mallory and Petes, 2000). Deletion of Sc*TEL1* severely impairs binding of ScEst1 and Est2, while absence of Mec1 results in a modest reduction in Est2's recruitment during G_1 (Goudsouzian et al., 2006). It has been suggested that increased number of ScRif2 molecules excludes Tel1 from longer telomeres, which would provide a mechanism for the preferential elongation of short telomeres (McGee et al., 2010; Teixeira et al., 2004).

Binding of ScMec1 to telomeres seems to be dependent on MRX. As in Sc*tel1* mutants, absence of either of MRX's components leads to short but stable telomeres, a phenotype that is not observed in fission yeast (Boulton and Jackson, 1998; Nakamura et al., 2002). Accordingly, ScMre11 binds to telomeres in late S phase. Deletion of Sc*MRE11* decreases binding not only of ScMec1, but also of ScEst1, Est2 and Cdc13 in late S

phase (Diede and Gottschling, 2001; Goudsouzian et al., 2006; Larrivée et al., 2004; Takata et al., 2005; Tomita et al., 2003). Accordingly, budding yeast MRX was shown to be required for new telomere addition (Nugent et al., 1998). However, in fission yeast, absence of both Rad3 and any of the MRN components results in complete resection of telomeres accompanied by chomosome-end fusions (Chahwan et al., 2003; Nakamura et al., 2002). This suggests that MRN plays a role at telomeres independently of Rad3's phosphorylation of Ccq1.

In addition to their role in telomerase recruitment, fission yeast Tel1 and Rad3 are also involved in telomere protection. Absence of both leads to G-overhang overextension, and Tpz1 and Ccq1 binding defects (Matsuura et al., 1999; Moser et al., 2009b). This is accompanied by association of Rad22 and Rhp51 to telomeres, and relaxation of TPE. Tel1 and Rad3 are not, however, the only DDR components ensuring telomere protection. Reconstitution of the G-overhang after DNA replication requires further processing of telomere ends. Early experiments showed evidence that G-overhangs are generated during late S phase (Wellinger et al., 1993b). G-overhangs disappear as a consequence of leading strand telomere replication (de Lange, 2002; Griffith et al., 1999; Makarov et al., 1997; Van Steensel et al., 1998). The absence of a single-stranded telomere region would presumably prevent ssDNA-binding proteins, such as Pot1, to associate with telomeres, and disrupt the t-loop conformation. Because of its role in 3'-overhang generation at DSBs, the MRN complex became a prime suspect for G-overhang formation. In fission yeast, Rad32 and Rad50 are required for G-overhang formation in *taz1*Δ cells (Hartsuiker et al., 2001; Tomita et al., 2004; Tomita et al., 2003). In budding yeast, the G-overhang is generated during late S phase, even in the absence of telomerase-mediated telomere addition (Dionne and Wellinger, 1996; Wellinger et al., 1996; Wellinger et al., 1993b). Concomitantly, fission

yeast's Nbs1 is recruited to telomeres during late S phase, suggesting that the G-overhang is also formed during late S phase in this model (Kibe et al., 2003).

According to this model, MRN-dependent 5'-resection should occur at telomeres during late S phase. This ensures binding of telomere components including telomerase, and the Pot1 and CST complexes. These results argue that binding of telomerase and G-overhang components to telomeres is dependent on 5'-resection by the MRN/X complex. Similarly, human hMRE11 and hNBS1 also bind to telomeres in late S phase, but also and particularly during G_2, coinciding with decreased hPOT1 binding (Verdun et al., 2005). MRN was also shown to co-localize to telomeres in HeLa cells (Zhu et al., 2000). In this case, hNBS1 associates with telomeres mainly in G_1, through interaction with TRF2. In contrast, hMRE11 and hRAD50 binding to TRF2 is persistent. Human telomeres were also found to be susceptible to enzymatic activity during G_2 phase, which increases in the absence of MRN. This suggests that MRN has a protective role at telomeres, when POT1 binding is diminished, or at least MRN competes with other agents of DNA-end processing (Verdun et al., 2005).

Telomeres elicit a DDR during G_2 in the form of ATM and MRN recruitment. However, no downstream effectors of a cell cycle checkpoint, such as mammalian Chk2 or p53, were found to be activated (Verdun et al., 2005). Experiments in fission yeast *taz1*Δ cells revealed that, DDR components such as Rad3, RPA and Rad26 are recruited to telomeres (Carneiro et al., 2010). However, this does not lead to Chk1 activation and cell cycle arrest. This was shown to be due to the exclusion of the DDR mediator Crb2, orthologue of human 53BP1. This exclusion depends on Pot1 and Ccq1, and is thought to involve specific changes in telomeric

heterochromatin (Churikov and Price, 2008; Denchi and de Lange, 2007; Guo et al., 2007; Tomita and Cooper, 2008).

In humans, hPOT1 inhibits checkpoint responses mediated by Rad3 orthologue ATR (Denchi and de Lange, 2007). However, in humans, Tel1 orthologue ATM can be recruited to telomeres without activating a full checkpoint response (Okamoto et al., 2013). In this case, hTRF2 inhibits a full checkpoint response independently, by preventing ATM localization to telomeres. However, if ATM is artificially recruited to telomeres, TRF2 blocks the association of the E3 ubiquitin ligase RNF168 to telomeres, preventing activation of 53BP1.

In budding yeast, it is ScRif1 that acts as a telomere checkpoint inhibitor is ScRif1, in a ScRap1-independent manner (Xue et al., 2011). This inhibition excludes Crb2 orthologue Rad9, RPA, and Rad26 orthologue Ddc2 from telomeres. However, in this model, ScRif1's function as a telomere checkpoint may be related to Rif1's inhibition of 5'-resection. This would prevent G-overhang extension, which could prevent binding of RPA.

These observations converge with the switch model proposed for telomere length regulation. In this model, telomeres would change from a "closed", protected state, to an "open", more permeable state that would result in telomeres being transiently recognized as DNA DSBs (Takata et al., 2005; Verdun et al., 2005). In fact, it was shown that Hydroxyurea (HU), a drug that stalls replication (Moser et al., 2009a), inhibits binding of Trt1, Pot1, Rad26 and RPA to fission yeast telomeres. Thus, it is arguable that the switch, which coincides with DNA replication, is triggered by passage of the replication fork through telomeres.

Thus, telomeres seem to be subject to several challenges during replication: 1) some Shelterin components are momentarily evicted by the DNA replication machinery and the t-loop is dismantled; 2) The lagging

strand-replicated telomere is resected, because of the end-replication problem; 3) The leading strand-replicated telomere becomes blunt-ended, because the G-overhang is complemented by replication of the opposing strand (Denchi and de Lange, 2007; Jacob et al., 2001; Makarov et al., 1997; Moser et al., 2009a; Muñoz-Jordán et al., 2001; Ohki et al., 2001; Watson, 1972; Wellinger et al., 1996; Wellinger et al., 1993b). These events can cause telomeres to shorten and the 3'-overhang to disappear or become too short for the t-loop conformation to be re-established (de Lange, 2002; Griffith et al., 1999; Makarov et al., 1997; Van Steensel et al., 1998). Subsequent alterations must be made in order for telomeres to maintain their canonical structure. The change in architecture to an open state during late S phase allows telomerase to access telomeres and add new telomere repeats, thus compensating resection. However, telomerase is unable to add telomere repeats to blunt ends because there is no G-overhang to which the RNA template can dock to (Lingner and Cech, 1996). Reconstruction of the G-overhang in the leading strand-replicated telomere by nucleases is conducted after its replication in late S phase (Diede and Gottschling, 2001; Larrivée et al., 2004; Makarov et al., 1997; Moser et al., 2009a; Tomita et al., 2003; Wellinger et al., 1993b). This generates an adequate substrate for both telomerase activity and binding of the single-stranded telomere binding proteins. The careful coordination of these mechanisms ensures telomeres maintain their structure after each cell replication.

Chapter 2 - Results

2.1 - Materials and Methods

2.1.1 - Mini-chromosome and Plasmid Construction. pFT2. Initial design and construction was performed by Miguel Godinho Ferreira. The *his3*$^{+}$ gene was cloned into pBluescriptII KS(+) using a blunt-ended NotI/SmaI digestion. A synthetic 100mer oligonucleotide containing subtelomeric sequences in a head-to-head orientation, derived from the pNSU70 plasmid (Sugawara, 1989) separated by an ApaI restriction fragment was cloned into the XbaI restriction site of *his3*$^{+}$. The resulting *his3*$^{+}$ fragment was then removed from pBluescriptII using PstI and SacI and cloned into the PstI and SacI sites of pREP3X. The SacI site was subsequently destroyed by digestion and blunt-end ligation to generate pREP-his-telo. The author performed the following cloning steps. An ApaI restriction fragment containing opposing telomere sequences separated by an *E. coli* kanR resistance gene was derived from the pEN53 plasmid (Nimmo et al., 1994) and cloned into the ApaI site, thus spacing the subtelomeric sequences of pREP3X-his-telo to give pFT2. Linearization of the plasmid was achieved by removing the SacI-enclosed kanR sequence in between the telomere sequences. pREP-*his3*$^{+}$. The *his3*$^{+}$ gene was cloned into pREP3X using PstI and SacI sites to generate pREP-*his3*$^{+}$. pREP42-*taz1*$^{+}$. pREP42- *taz1*$^{+}$ was used in the construction of the taz1+ o/e strain. The *taz1*$^{+}$ORF was cloned in a pTOPO vector (Invitrogen). pTOPO was then digested with BamHI and SalI and the resulting *taz1*$^{+}$fragment was cloned into the BamHI and SalI sites of pREP42 vector.

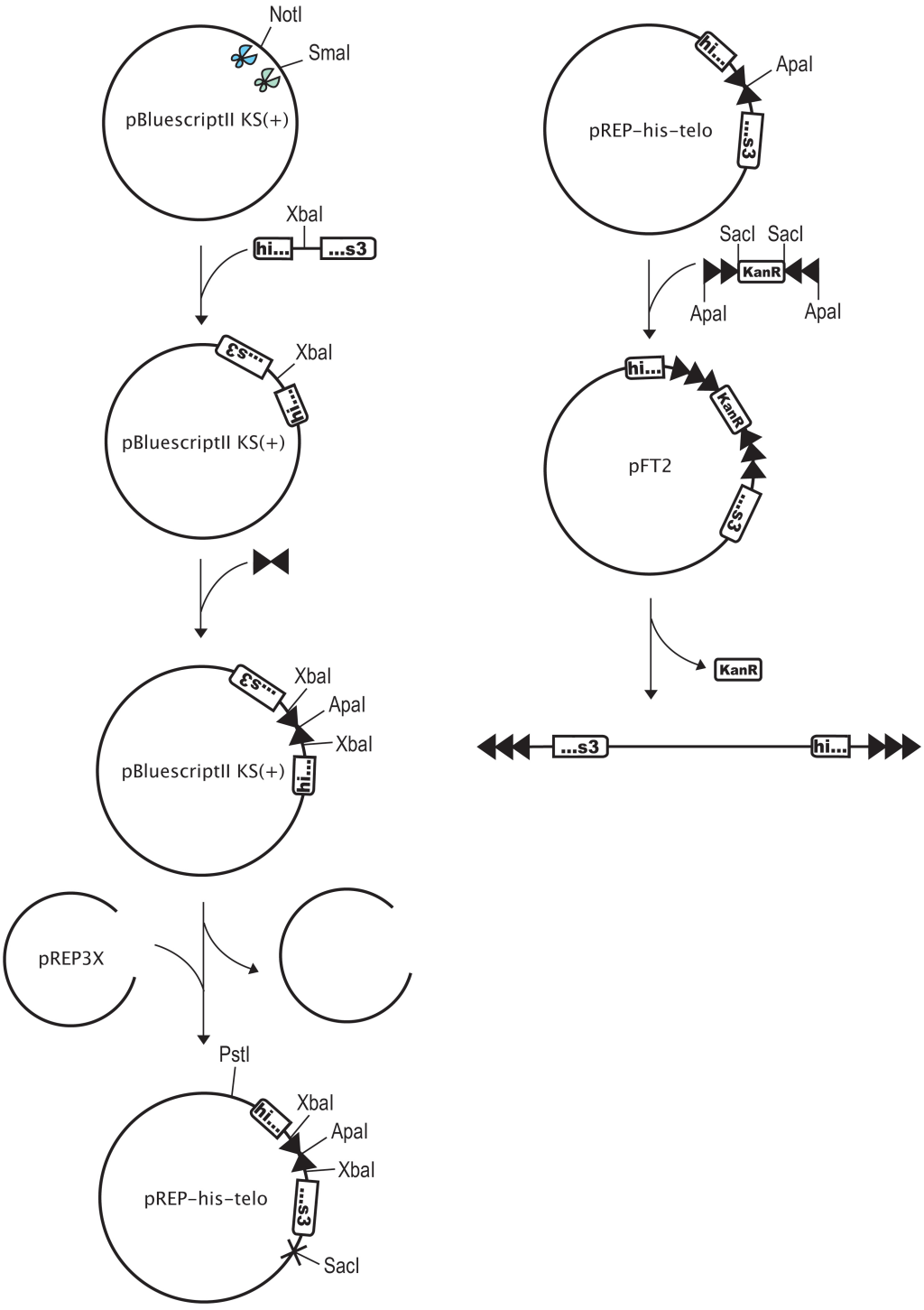

NotI
SmaI
pBluescriptII KS(+)
XbaI
hi...
...s3
ES...
XbaI
hi...
pBluescriptII KS(+)
XbaI
ApaI
XbaI
ES...
hi...
pBluescriptII KS(+)
pREP3X
PstI
XbaI
ApaI
XbaI
hi...
...s3
SacI
pREP-his-telo
hi...
ApaI
...s3
pREP-his-telo
SacI
SacI
KanR
ApaI
ApaI
hi...
KanR
ES...
pFT2
KanR
...s3
hi...

Figure 2.1. Construction of the pFT2 plasmid. Restriction enzymes used during cloning are indicated when required. Black arrows represent telomeric and subtelomeric sequences.

2.1.2 - Media and Genetic Methods. Standard recipes for rich media (Yeast Extract with Supplements — YES) and Edinburgh minimal media (EMM) were used as described in (Moreno et al., 1991), except when otherwise noted.

General media composition:

- Edinburgh Minimal Medium (EMM): 3 g/l potassium hydrogen phthallate, 2.2 g/l Na2HPO4, 20 ml/l salts (stock x 50), 1 ml/l vitamins (stock x 1000), 0.1 ml/l minerals (stock x 10,000).
- Yeast extract with supplements (YES): Yeast extract (YE: 0.5% (w/v) Oxoid yeast extract, 3.0% (w/v) glucose) + 225 mg/l adenine, arginine, histidine, leucine, uracil and lysine hydrochloride.
- Malt extract (ME): 3% (w/v) Bacto-malt extract (Difco). Supplements added as for YES, except lysine. Adjusted to pH 5.5 with NaOH.
- Supplements: Adenine, Leucine, Histidine, Uracil and NH4 stocks prepared at 7.5 g/l (14 ml added to EMM) and Uracil at 2.5g/l (42 ml added to EMM).
- Salts x 50: 52.5 g/l MgCl2.6H20, 0.735 mg/l CaCl2.2H20, 50 g/l KCl, 2 g/l Na2SO4.
- Vitamins x 1000: 1 g/l pantothenic acid, 10 g/l nicotinic acid, 10 g/l inositol, 10 mg/l biotin.
- Minerals x 10.000: 5 g/l boric acid, 4 g/l MnSO4, 4g/l $ZnSO4.7H_2O$, 2 g/l $FeCl_2.6H_2O$, 0.4 g/l molybdic acid, 1 g/l KI, 0.4 g/l $CuSO_4.5H_2O$, 10 g/l citric acid.

For EMM, supplements were added after autoclaving. 2% (w/v) Glucose was added from a filtered stock of 40% (w/v) Glucose.

Solid media was made by adding 2% Difco Bacto Agar. All media was prepared in bulk. Sterilization was made by autoclaving at 10 psi for 20 min. The media was stored in 500 ml bottles and agar was remelted in a microwave oven before using.

Fission yeast growth conditions were as described in (Moreno et al., 1991), except when otherwise noted. Liquid cultures were grown in YES or *EMM media* at 32ºC with appropriate shacking, except when otherwise noted. Generation of knock-out fragments was performed as described in (Bähler et al., 1998). Transformation of fission yeast was performed as described in (Moreno et al., 1991). Using the lithium acetate protocol for yeast transformation, cells were incubated overnight in liquid YES media. Before transformation, these cultures were diluted and incubated until they were exponentially growing. Cells were pelleted and washed once in 1ml of LiAc-TE (0.1 M Lithium acetate, 10 mM Tris pH 7.5, 1 mM EDTA). Cells were re-suspended in 1 ml of LiAc-Te. From this suspension, 100ml were retrieved for any single transformation, and mixed with pre-boiled 10ml of carrier salmon sperm DNA (10 mg/ml, Stratagene), and 1mg-1.5mg of transforming DNA (either knockout fragment or plasmid DNA). The mix was incubated for 5 minutes at RT. 260 ml of LiAc-TE-PEG (LiAc-TE plus 40% PEG4000) was added to the mix. Cells were incubated for 45 minutes at 32ºC. A volume of 43 ml of DMSO (Sigma) was added and mixed gently. Cells were then heat-shocked for 7 minutes at 42ºC. Cells were pelleted for 1 minute at 3000 rpm, washed once with YES and let to recover overnight at 32ºC (for the case of Insertions or knockouts) or plated directly in selective media (In the case of plasmid DNA). Correct integration was confirmed by PCR analysis.

Tetrad dissection and mass sporulation for the generation of mutant strains was performed as described in (Moreno et al., 1991). Tetrad dissection for the generation of the *trt1Δ* strain containing pFT2 was performed in YES media. The dissected tetrads were then incubated overnight at 25ºC. Once germination occurred, each spore was singularly transferred to EMM media lacking Leucine, to select for offspring containing the pFT2 plasmid. Mass sporulation was conducted to generate the remaining mutant strains containing the pFT2 plasmid. After mating, spores were selected in water containing 0.5% *Helix pomatia* (snail) digestive juice. These were germinated for 7-9 hours in liquid YES media, and then plated in EMM media lacking Leucine.

2.1.3 - Strains. Strains used in this study are described in Table 1. Lines derived from genetic crosses are indicated in the parental column with both parental strains indicated. All deletions were performed using the procedures described in (Bähler et al., 1998). *taz1*$^{+}$ *o/e*. To create strain MGF1898, pREP42-*taz1*$^{+}$ was digested with KpnI and transformed for integration. Insertion at the *taz1*$^{+}$ locus was confirmed by PCR.

2.1.4 - Primers. Primers used for amplification and sequencing of captured fusions were as follows: 495:GAACTTCAGCCTTATCGCTG; 496: CCACGGAAATAACCGAACCA; 613: GGGTAATAATTGATATGAGGGC; 614: CCACGGAAATAACCGAACCA. Primers used for confirmation of insertion of pREP42-*taz1*$^{+}$ fragment at the *taz1*$^{+}$ locus were as follows: 178: TTCGCCTCGACATCATCTGC; 375: CCTCAGTGGCAAATCCTAAC; 711: TCTTTTACAGTTTCCTTCCC; 717:ATTGCAGAGTAAACACGACG; 736: TGGACTTTGCGTATGAGACG

2.1.5 - Serial dilution assays. Cultures were grown at 32ºC to logarithmic phase and re-suspended to a cell density of $1x10^7$ cells/ml. 10-fold serial dilutions were performed in EMM media and 5 µl of each dilution were spotted onto EMM plates with the described supplements and incubated at 32ºC up to 7 days. Serial dilutions for Figure 2.27 were performed similarly, but were plated in 2 µM Camptothecin-, 8mM Hydroxyurea-supplemented EMM media, or subjected to 200J/m2 (UV Stratalinker 1800, Stratagene), as previously published (Williams et al., 2008).

2.1.6 – Determination of doubling times. Cell cultures were grown at 32ºC overnight, then diluted and followed for 10 hours in logarithmic phase in EMM media containing or lacking leucine. Doubling times were calculated from growth rate through the formula T_d= log(2)/log(1+r). where *r* stands for growth rate.

2.1.7 - Telomere fusion assays. Cultures grown at 32ºC to logarithmic phase in EMM media lacking Leucine and plated on solid EMM media lacking Leucine ± Histidine. Plates were incubated at 32º C up to 7 days and colonies were counted. Cell densities were determined by counting using a hemacytometer. For time course experiments, cultures were diluted at each time point to $5x10^5$ cells/ml. At the designated times, 8.000 cells were plated in –LEU +HIS EMM media, and 80.000 cells were plated in –LEU –HIS EMM media. For determining the frequency of spontaneous fusion events, 10.250 cells were plated in –LEU +HIS media, and 205.000 cells were plated in –LEU –HIS media, using 15 cm diameter petri dishes.

2.1.8 - Western blotting. Cells were lysed in 20% TCA and the resulting protein extracts were resolved by SDS-PAGE, transferred to PVDF membranes (GE Healthcare RPN203D) and probed with primary α-Taz1

antibody (a gift from Julia P. Cooper). After incubation with HRP-conjugated secondary antibodies (GE Healthcare NA934), bands were visualized using ECLPlus (GE Healthcare RPN2132) in a STORM scanner (Amersham). Quantification was performed using ImageJ software.

2.1.9 - Southern blotting. Genomic DNA was obtained from exponentially growing cells in YES or EMM media with supplements, using the phenolic extraction method described (Moreno et al., 1991) and digested with the appropriate restriction enzymes. Southern blot analysis was performed as described (Nakamura et al., 1997). Briefly, DNA was separated in 0.8% agarose gels. Each gel was subsequently incubated in 0.25N HCl (10 ml HCl in 500 ml H_2O) for 15 minutes, followed by 30 minutes incubation in Blot 1 (20g NaOH, 87.6g NaCl in 1 litter of H_2O), and 1-hour incubation in Blot 2 (77g NH4Ac, 0.8 NaOH in 1 litter H_2O). After this treatment, DNA was transferred by capillarity from the agarose gels to genomic blotting membranes (Bio-Rad, #162-0196). The DNA was then cross-linked using UV radiation and the membranes were hybridized using Church-Gilbert solution (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M Na_2HPO_4, 4 ml H_3PO_4 85%, up 1 litter with H_2O) at 65ºC. An overnight incubation was performed with a telomere repeat probe or a *rad4*$^+$ genomic probe labeled with 32P using the Prime-it II random primer labeling kit (Stratagene). Probes were prepared as follows: 25 ng of DNA template, 10 ml of random oligo primers and 23 ml of H_2O were mixed and heat-boiled for 5 minutes. The mixture was centrifuged down and 10 ml of 5x dCTP buffer, 1 ml of (-) Exo Klenow (Stratagene) and 2.5 ml of radioactively labelled dCTP nucleotides were added. The reaction was incubated at 37ºC for 15 minutes. The membrane was washed for 30 min at 65ºC with a 1X SSC 1% SDS solution and exposed to a PhosphoImager screen (Amersham) for 1 to 3 days depending on signal strength. The PhosphoImager screen was scanned

with a STORM scanner (Amersham). Telomere length was calculated by normalizing molecular weight with telomere signal intensity, as described in (Kimura et al., 2010). Number of pFT2 copies per cell was calculated by dividing signal intensity from *ars1* fragment present in pFT2 with the signal intensity from the endogenous *ars1*. This number was multiplied by a factor of 0.3 to account for the measured fraction of cells in a population under selection for *LEU2* harboring pFT2.

2.1.10 - PCR reactions and sequencing. pFT2 end fusions were amplified by PCR. Forward primers (495: GAACTTCAGCCTTATCGCTG; 613: GGGTAATAATTGATATGAGGGC) were used for promoter proximal sequencing and the reverse primers (496: CCACGGAAATAACCGAACCA; 614: CCACGGAAATAACCGAACCA) for terminator proximal sequencing. Genomic DNA for sequencing of *trt1Δ* fusions was obtained by colony PCR. Briefly, a sample of a histidine-producing colony isolated in solid media was mixed with Z buffer (2.5 mg/ml of Zymolase, 1.2 M Sorbitol, 0.1 M Sodium phosphate pH 7.4 and a 1:10 of 10x Lyzing enzymes stock 100 mg/ml). This mix was incubated in a thermocycler for 30 minutes at 30ºC followed by 5 minutes at 100ºC. Following amplification, DNA from the PCR reaction was separated by gel electrophoresis and purified using the Wizard SV Gel and PCR clean up system (Promega).

For DNA sequencing, 90 ng of the purified PCR DNA were added to 2 ml of buffer, 2 ml of Terminator Ready Reaction Mix (BigDye® Terminator v1.1 Cycle Sequencing Kit), 1 ml of primer, 0.5 ml DMSO (Sigma) and up to 10 ml of deionized water. DNA amplification for sequencing was as follows: 96ºC 1 min, followed by 25 cycles of 96ºC 10 sec, 50ºC for 5 sec and 60ºC for 4 min. The sequencing reaction was precipitated for a minimum of 3 hours after mixing with 10 ml of H_20, 2 ml of 3 M Sodium Acetate pH4.6 and 50 ml of 95% ethanol. This mix was centrifuged for 30 minutes at

14000 rpm 4ºC. The supernatant was carefully aspirated and the pellet was washed with 250 ml of 70% cold ethanol and centrifuged again for 15 minutes. Finally, the supernatant was carefully aspirated and the pellet dried at 37ºC.

Genomic DNA for sequencing pFT2 fusions in *taz1*$^{+}$ *o/e* strains was either obtained after colony isolation by phenolic extraction, or purified from frozen fission yeast pellets by STABVIDA. All sequencing reactions were performed by STABVIDA, using the primers described previously.

Table 1. Strains used in this work

Strain	Genotype	Creator
MGF10	*h- ade6-M210 his3-D1 leu1-32 ura4-D18*	R. McIntosh
MGF11	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18*	R. McIntosh
MGF21	*h+ taz1::kanMX6*	J Cooper
MGF129	*h+ ade6-M210 ura4-D18 leu1-32 his3-D1 pku70::kanMX6*	P Baumann
MGF479	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 rad32::kanMX6*	C . Reis
MGF803a	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 nbs1::natMX6*	C. Reis
MGF816	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 rad16::kanMX6*	This study
MGF1014	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 lig4::natMX6*	This study
MGF1015	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 lig4::natMX6*	This study
MGF1368	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 ctp1::KanMX6*	C Reis
MGF1405	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 rad50::hphMX6*	C Reis
MGF1449	*h+ pot1::pot1ts-kanMX*6	C. Pitt
MGF1478	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pot1::pot1ts-kanMX6*	T. D. Carneiro
MGF1518	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pREP42-taz1-GBD*	This study
MGF1519	*h+ ade6-M216 leu1-32 ura4D-18 taz1::Kan pREP42-taz1-GBD*	This study
MGF1532	*h+ ade6-M210 his3-D1 leu1-32 ura4-D18 taz1::kanR pFT2*	This study
MGF1545	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2*	This study
MGF1546	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pLEU2-his3+*	This study
MGF1547	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 (circular)*	This study
MGF1610	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-*	This study

	taz1'	
MGF1613	*h+ ade6-M210 his3-D1 leu1-32 ura4-D18 taz1::kanR pFT2 pREP42-taz1+*	This study
MGF1698	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 ctp1::KanMX6*	C. Reis
MGF1885	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 rad50::kanMX6*	C . Reis
MGF1898	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+*	This study
MGF1899	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ lig4::natMX6*	This study
MGF1900	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 rad16::kanMX6*	This study
MGF2022	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 trt1::hphMX6*	This study
MGF2023	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 trt1::hphMX6*	This study
MGF2033	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 rad50::kanMX6*	This study
MGF2037	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 nbs1::natMX6*	This study
MGF2038	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 rad50::kanMX6*	This study
MGF2039	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 ctp1::kanMX6*	This study
MGF2040	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 rad32::kanMX6*	This study
MGF2063	*h+ ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1 ku70::kanMX6*	This study
MGF2099	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 trt1::ura4+*	This study
MGF2100	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 trt1::ura4+ pFT2*	This study
MGF2108	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2*	This study
MGFT1	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ trt1::hphMX6*	This study
MGFTL4	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ lig4::natMX6 trt1::hphMX6*	This study
MGFTR16	*h+ ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ rad16::kanMX6*	This study
MGFTR32	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ rad32::kanMX6 trt1::hphMX6*	This study
MGFTR50	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ rad50::kanMX6*	This study
MGFTN1	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ nbs1::natMX6 trt1::hphMX6*	This study
MGFTC1	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ ctp1::kanMX6*	This study
MGFFT2K	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2-KanMX6*	This study
MGFFT2P	*h+ ade6-M216 his3-D1 leu1-32 ura4-D18 pot1::pot1ts-*	This study

	kanMX6 pFT2	
MGFFT2C	*h- ade6-M216 his3-D1 leu1-32 ura4-D18 ccq1::hph pFT1*	This study
MGFPRPOT	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-pot1+-GBD*	This study
MGFPRCCQ	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-ccq1+-GBD*	This study
MGFPRTAZ	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-taz1+-GBD*	This study
MGFPRRAP	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-rap1+-GBD*	This study
MGFPRGBD	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 pREP42-GBD*	This study
MGFFT2C	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 pFT2 taz1::pREP42-taz1+ (circular)*	This study
MGFPRLIG	*h- ade6-M210 his3-D1 leu1-32 ura4-D18 lig4::hph pFT2 pREP42-taz1+*	This study

2.2 - Characterization of a telomere fusion capturing assay

2.2.1 - General aim

The main goal of this work was to study telomere dysfunction and the DNA repair events that follow. The fission yeast *Schizosaccharomyces pombe* was chosen as a model organism for several practical reasons. Firstly, it is a fast growing unicellular eukaryote, of easy genetic manipulation. Secondly, it contains only three chromosomes, and it can survive critical telomere dysfunction by circularizing all of them (Nakamura et al., 1998). These features elect fission yeast as a privileged system for investigating events following telomere deprotection.

I was proposed to develop a positive selection assay to capture telomere fusion events. This assay would be based on a linear plasmid with telomere sequences, originally conceived by Miguel Godinho Ferreira.

Linear plasmids have been used for the study of various properties of telomere biology, particularly in budding yeast. In these studies, plasmids are successfully redesigned into linear episomes by cloning telomere sequences onto each tip. One groundbreaking publication demonstrated that sequence repeats from Tetrahymena's telomeres, which differ from budding yeast telomere sequences, were able to stabilize a linear plasmid in budding yeast (Szostak and Blackburn, 1982). Surprisingly, these sequences promoted the addition of new budding yeast telomere repeats to the ends of the linear plasmid (Shampay et al., 1984). Similar results were obtained by cloning telomere sequences from other organisms into a budding yeast plasmid, such as *Oxytricha fallax* and humans (Brown, 1989; Pluta et al., 1984). These early studies confirmed the evolutionarily conserved nature of telomere protection, while strongly hinting at the mechanism through which chromosome ends bypass the end-replication

problem. Other publications, using linear plasmid-based strategies, focused on varied aspects such as the effect of telomeres in the stability of budding yeast episomes (Dani and Zakian, 1983), the specific sequence requirements for new telomere addition at plasmid ends (Murray et al., 1988), the role of Rap1 telomere binding in chromosome-end protection (Lustig et al., 1990; McEachern et al., 2000; Wiley and Zakian, 1995), telomere length regulation (Runge and Zakian, 1989; Wiley and Zakian, 1995), and G-overhang generation (Dionne and Wellinger, 1998; Wellinger et al., 1993b). Perhaps one of the most significant discoveries using linear plasmids is the discovery of the first *est* mutant in budding yeast (Lundblad and Szostak, 1989).

At least one study shows that fission yeast plasmids can also be stably linearized by the addition of telomere sequences (Nimmo et al., 1994). One of the advantages of resorting to linear plasmids in studying telomere biology is that they can report on events, which could otherwise be lethal, should they occur at chromosomes.

2.2.2 - Design of a telomere fusion-capturing assay

The plasmid-based assay was developed to report telomere fusions. This was possible due to an engineered selectable marker, that is only expressed when such fusions occur. For this, we placed a construct containing 258 bp telomere repeats in a head to head arrangement, inside the second intron of the *his3*$^{+}$ marker. Introns are recognized by specific consensus 5' and 3' splice sites. Therefore, introduction of an exogenous sequence into an intron of *his3*$^{+}$ should not disrupt its function (De Conti et al., 2012). The telomere construct was flanked by 80 bp of subtelomeric sequences and a KanMX6 resistance gene separated the telomere repeats. The modified *his3*$^{+}$ gene was cloned in a fission yeast plasmid

carrying a *LEU2* marker, which would serve as base selection for plasmid maintenance (Maundrell, 1993). This allowed for selection in media lacking both leucine and histidine (see *Materials and Methods*). We named this plasmid pFT2 (after Fusion Trap, version #2; Figure 2.1). The telomere sequences did not disrupt *his3*$^{+}$ expression, and transformed cells could grow in media lacking leucine and histidine (pFT2-Cir, Figure 2.2). Removing the KanMX6 sequence by enzymatic digestion generates a linear molecule. Transforming the linearized pFT2 into WT cells created a linear episome in which the *his3*$^{+}$ gene was disrupted, split between the two extremities of the plasmid (Figure 2.2).

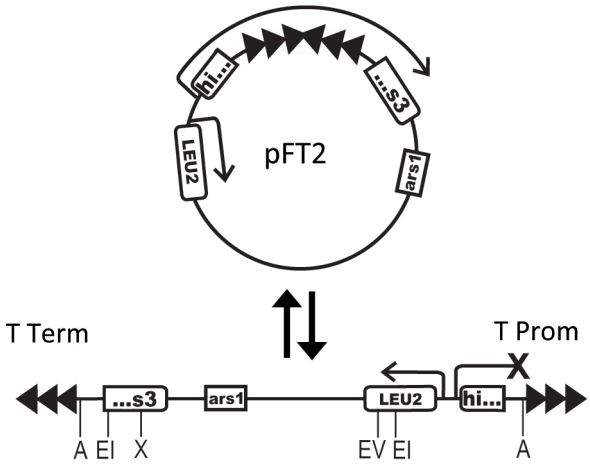


Figure 2.2. Telomere fusion capturing assay used to detect telomere fusions. Fusion of both ends of the linear pFT2 plasmid generates a circular episome that expresses the *his3*$^{+}$ gene. Relevant restriction sites are indicated as follows: A- ApaI; EI- EcoRI; EV- EcoRV; X- XhoI

As predicted, this version of pFT2 still conferred growth in the absence of leucine, but not in media lacking histidine (pFT2-Lin, Figure 2.3).

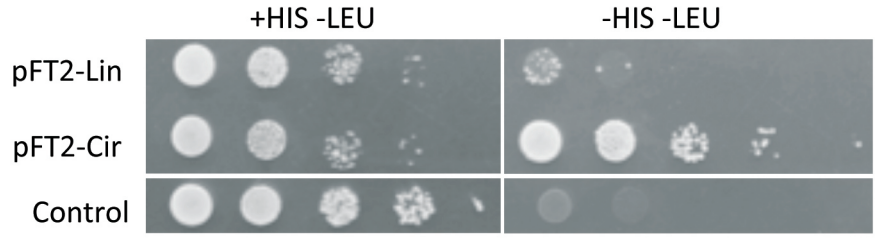


Figure 2.3. *his3*$^{+}$ expression in strains carrying the pFT2 plasmid. Tenfold serial dilutions of WT cells carrying either the linear (pFT2-Lin) or circular (pFT2-Cir) plasmid were plated on permissive (+HIS -LEU) or restrictive (-HIS -LEU) media. Control cells contain a *LEU2*-based plasmid that lacks the *his3*$^{+}$ gene.

In principle, should the plasmid ends fuse, the reconnected *his3*$^{+}$ halves would be able to produce a full transcript. Thus, pFT2 could be used to capture any end-fusion reactions resulting from telomere dysfunction, just as long as these would not disrupt the *his3*$^{+}$ coding sequence. Increase in intron length should not pose a restriction to detecting telomere fusions, as cells harboring a circular version of pFT2 carrying the 1.3 Kb KanMX6 cassette in the *his3*$^{+}$ intron were still able to grow in media lacking histidine (Figure 2.4).

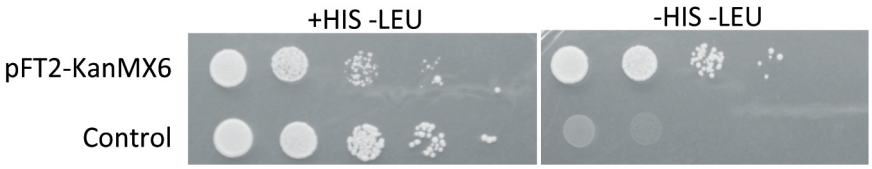


Figure 2.4. Increasing intron length does not compromise expression of *his3*$^{+}$. Tenfold serial dilutions of WT cells carrying the circular plasmid with a KanMX6 cassette in between telomere repeats (pFT2-KanMX6), plated on permissive (+HIS -LEU) or restrictive (-HIS -LEU) media. Control cells contain a *LEU2*-based plasmid that lacks the *his3*$^{+}$ gene.

To test whether linearized pFT2 can be transformed as a stable, linear episome, we analyzed the ability of several transformants to grow in histidine-depleted media. 6 out of 50 (12%) WT colonies transformed with pFT2 in its linear configuration were unable to grow in the absence of histidine. However, the remaining colonies (78%) were able to grow in the same conditions. As telomere sequences are treated as DNA DSBs if left unprotected, it is likely that naked pFT2 molecules attracted the DNA repair machinery before the assembly of Shelterin could take place, in most transformations. As a result, the plasmid ends fused, reestablishing *his3*$^{+}$ function.

Southern blot analysis was conducted to determine if the candidates which did not grow in the absence of histidine maintained a linearized pFT2. Genomic DNA was digested using two restriction enzymes, EcoRV and XhoI. If pFT2 was kept in a linear conformation, these two enzymes should separate the plasmid ends in two distinct fragments of approximately 2 Kb and 0.9 Kb, respectively (Figure 2.2). Probing for telomere repeats identified 2 candidates (4%) with the expected telomere pattern (Figure 2.5). Either single digestion produces one telomeric smear and an upper band corresponding to the undigested plasmid end. In this

case, the undigested plasmid end is superimposed with the endogenous telomere fragments (Figure 2.5, forth and fifth lanes). With the double digestion, two independent smears are observed, corresponding to both plasmid ends (Figure 2.5, sixth lane). Thus, pFT2 is maintained in a linear conformation. Furthermore, the telomeric smears are suggestive of telomerase activity at the plasmid ends, which would generate diversity in telomere sizes by adding new repeats (Blackburn and Gall, 1978; Blackburn and Greider, 1985; Emery and Weiner, 1981; Johnson, 1980; Shampay et al., 1984; Teixeira et al., 2004).

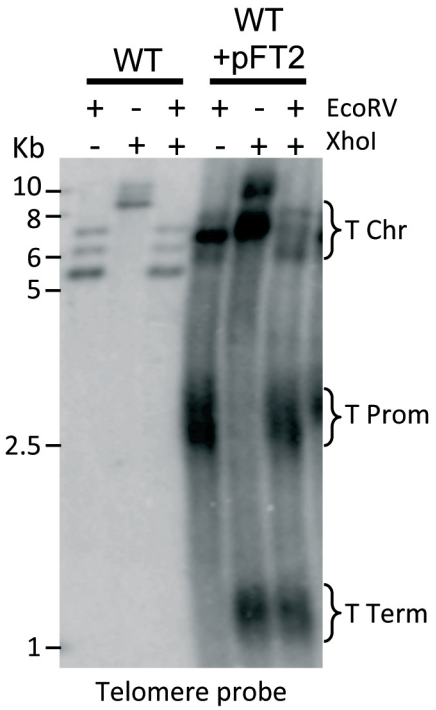


Figure 2.5. Southern blot analysis of telomeres in a WT strain harboring pFT2. Genomic DNA extracts were digested with EcoRV and/or XhoI enzymes and probed for telomeres (see Fig. 2.11 for details). Promoter-proximal (T Prom), terminator-proximal telomeres (T Term) and chromosomal telomeres (T Chr) are depicted.

It was not known whether the addition of extrachromosomal telomeres would affect fission yeast viability. Therefore, I monitored the effect of pFT2 in the growth profile of a WT strain (Figure 2.6). Doubling time for a strain carrying a control plasmid was 7 hours, twice of what is

expected from a WT strain bearing no plasmids. Compared to a control plasmid, neither configuration of pFT2 caused significant growth changes in selective or permissive media, suggesting that extrachromosomal telomeres do not impair fission yeast viability.

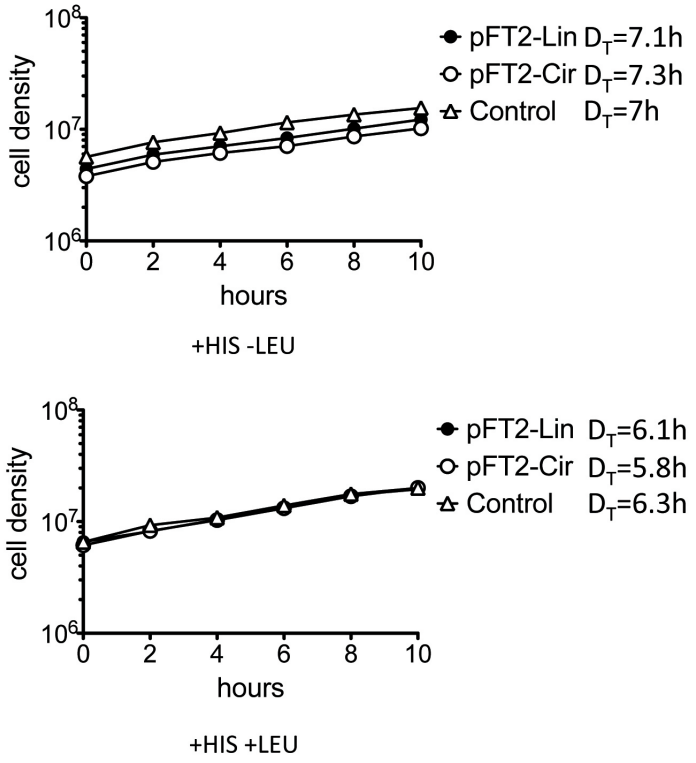


Figure 2.6. pFT2 does not alter growth rate in WT strains. Liquid cultures of WT cells carrying the linear (pFT2-Lin), the circular (pFT2-Cir), or a control plasmid were followed for 10 hours in +HIS-LEU and +HIS +LEU minimal media. Samples were retrieved every 2 hours to determine cell density. T_D designates doubling times for each culture.

In addition, cloning telomere repeats into a plasmid could alter its segregation. Traditional yeast plasmids are quickly lost in dividing cultures,

since they do not possess a centromere to ensure equal segregation during mitosis. This has also been observed for linear plasmids in budding yeast (Szostak and Blackburn, 1982). Nevertheless, adding telomere repeats to budding yeast circular plasmids was shown to improve their stability (Longtine et al., 1992). This was not the case for fission yeast since, in the absence of selection, both versions of the pFT2 plasmid were lost following a profile similar to a control plasmid (Figure 2.7). Surprisingly, only 29%-60% of cells in liquid culture maintained plasmids, even under selection. In this regard, the loss rate of pFT2 was determined by a half-life of 3,28±0,18 generations (SEM n=3) for its linear configuration and 3,21±0,14 generations (SEM n=3) for its circular configuration. This was higher than the control plasmid, which has a half-life of 4,32±0,20 generations (SEM n=3).

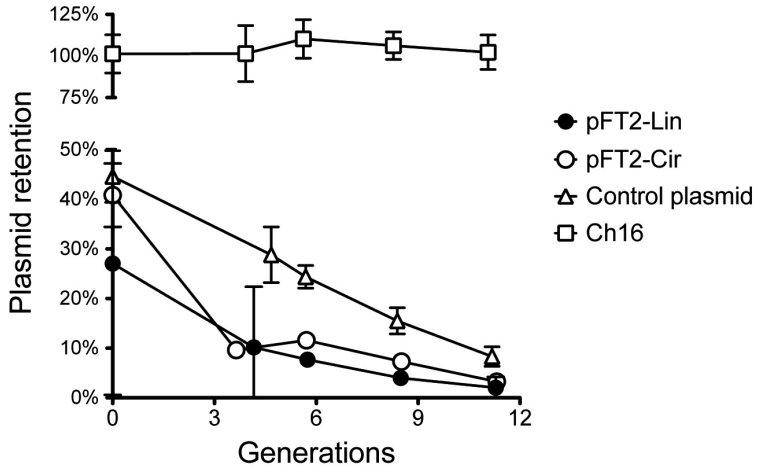


Figure 2.7. pFT2 is gradually lost from cells in the absence of selection. WT cells carrying the linear (pFT2-Lin), the circular (pFT2-Cir), a control plasmid, or the Ch16 artificial chromosome (Ch16) were plated daily on -LEU (pFT2-Lin, pFT2-Cir, Control), –ADE (Art. chrom.), and Complete minimal media. Frequencies were calculated by dividing the number of colonies growing in complete minimal media and –LEU/-ADE. Error bars represent SEM of 3 replicates for each time point.

Thus, cloning telomere repeats into a fission yeast plasmid exert an observable negative effect in segregation. Moreover, plasmid retention under selection seems to be different for all tested plasmids (Figure 2.8).

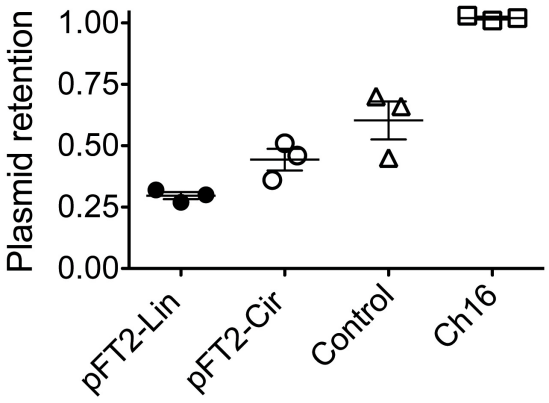


Figure 2.8. Steady-state retention of the pFT2 plasmid in a population of WT cells. Cells were grown in +HIS -LEU liquid media and then plated in +HIS -LEU and +HIS +LEU media in triplicate. Percentage of leu+ cells in the population was determined by dividing the number of leu+ colonies by the total number of colonies. Error bars represent SEM of 3 independent experiments. Plasmid retention values for pFT2-Lin suggest a statistically significant difference when compared with other tested plasmids (p-values for pFT2-Cir: 0.0342 and Control plasmid: 0.0177), but t-test failed to detect a difference between pFT2-Cir retention and the Control plasmid (p-value: 0.1473).

However, the differences between the closed version of pFT2 and the control plasmid were not statistically significant, which limited further interpretation. Differences in steady-state plasmid maintenance for both configurations of pFT2, seem to be more pronounced in the presence than in the absence of selection. It is possible that a disadvantage in segregation conferred by telomere repeats is more pronounced when telomeres are terminal, than when they are internal.

In conclusion, a new positive detection assay for telomere fusions was developed, consisting of a linear plasmid maintained by telomere sequences at each end. Initial characterization suggested that this construct, named pFT2, was innocuous to fission yeast cells. pFT2 was predicted to react to telomere dysfunction by expressing a marker gene.

2.2.3 - A quantitative method for detecting chromosome-end fusions

To assess whether the above-described assay would capture chromosome-end fusions, three proof of principle experiments were performed. These encompassed known scenarios of telomere dysfunction: gradual erosion of telomeres, immediate resection of chromosome ends, and chromosome-end fusions without loss of telomere sequences.

Gradual erosion of telomeres was modeled by deleting the telomerase *trt1*$^{+}$ gene. A WT strain carrying pFT2 was crossed with an early generation telomerase mutant. Their haploid offspring were monitored in liquid culture over time, as they underwent telomere erosion. Culture samples were harvested for 12 consecutive days for Southern blotting and plated in solid media in the presence and absence of histidine. In this way, the frequency of histidine-producing colonies, and, consequently, of plasmid-end fusions, would be calculated at each time point. Similar to what was previously published (Nakamura et al., 1998), cell density profiles were able to discern crisis and recovery of the *trt1Δ* population (Figure 2.9).

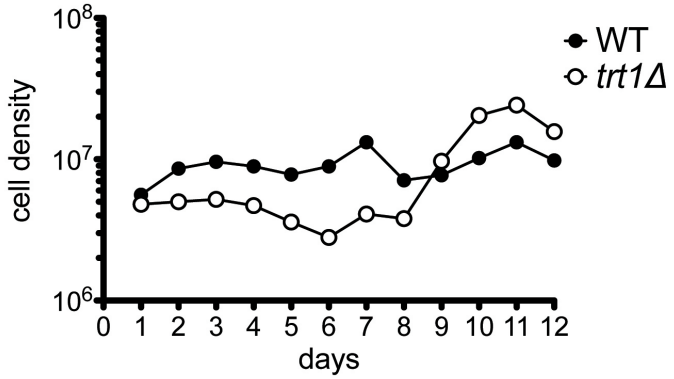


Figure 2.9. Growth profiles of WT and *trt1Δ* strains. Cells were grown in liquid +HIS -LEU media for 12 days. Each day, cell density was determined by counting in a hemacytometer, and the cells were diluted into 20 mL of fresh media at 5 x 10^5 cells/mL.

Probing for telomere repeats in a Southern blot analysis demonstrated the gradual erosion of telomeres in the *trt1Δ* culture (Figure 2.10).

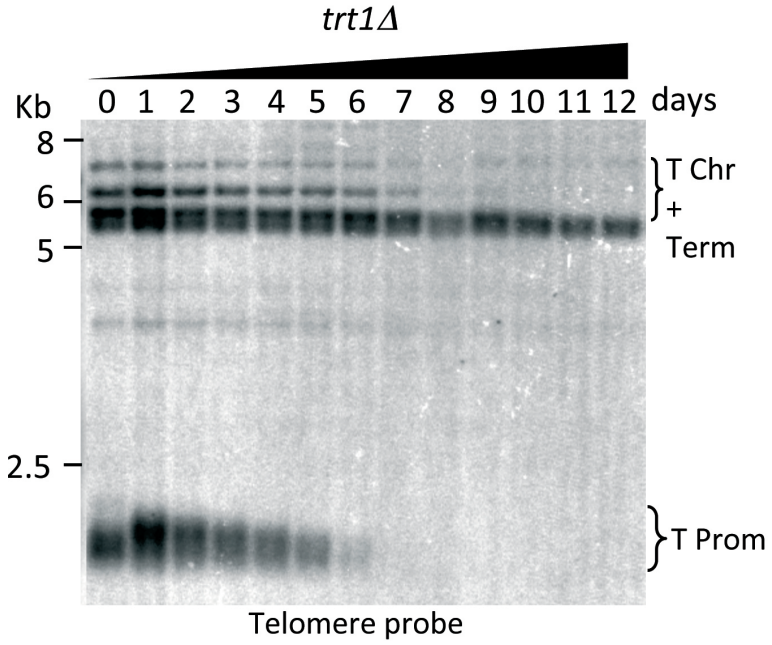


Figure 2.10. Southern blot analysis of telomere shortening in a *trt1Δ* strain. Samples retrieved from the cultures depicted in figure 2.9 and genomic DNA extracts were digested with EcoRV. Promoter-proximal (T Prom), terminator-proximal telomeres (T Term) and chromosomal telomeres (T Chr) are depicted. EcoRV digestion did not permit analysis of erosion at the T Term end.

While the WT strain did not grow in the absence of histidine, the *trt1Δ* mutant gave rise to *his3*$^{+}$-producing colonies (Figure 2.11). These increased in frequency up to 6% from day 6 onwards, coinciding with the lowest signal of plasmid telomeres detected by Southern blotting. This frequency is most likely an underestimation, since fusions are also likely to occur in ways that do not reestablish proper expression of the *his3*$^{+}$ marker.

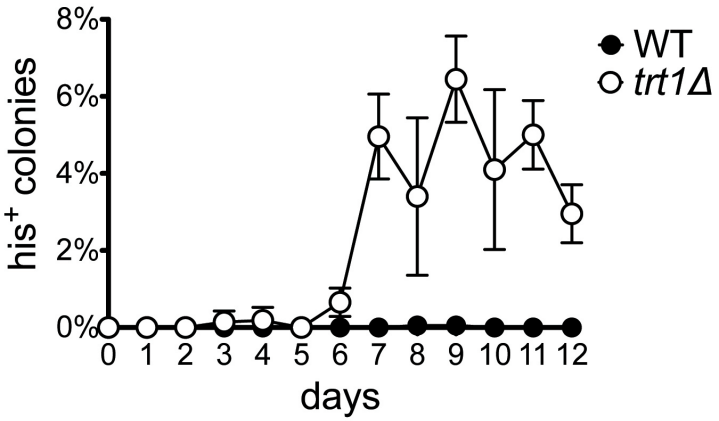


Figure 2.11. Frequency of WT and *trt1Δ* strains able to express histidine. Samples retrieved from the cultures depicted in figure 2.9 were plated in solid media. Percentages were calculated by dividing the number of colonies growing in +HIS -LEU and -HIS -LEU. Error bars represent SEM of 3 replicates for each time point.

Using the same procedure, the ability to detect fusions after telomere erosion was further tested in a *ccq1Δ* mutant (Figure 2.12). pFT2 was unable to detect fusions in this strain. It is known that *ccq1Δ* telomeres maintain very short telomeres via HR-mediated mechanisms (Tomita and

Cooper, 2008). Thus, it is likely that the ongoing HR either maintains telomere sequences, preventing the plasmid from fusing, or that it simply destroys the *his3*$^{+}$ coding sequence. However, this hypothesis was not investigated via Southern blotting.

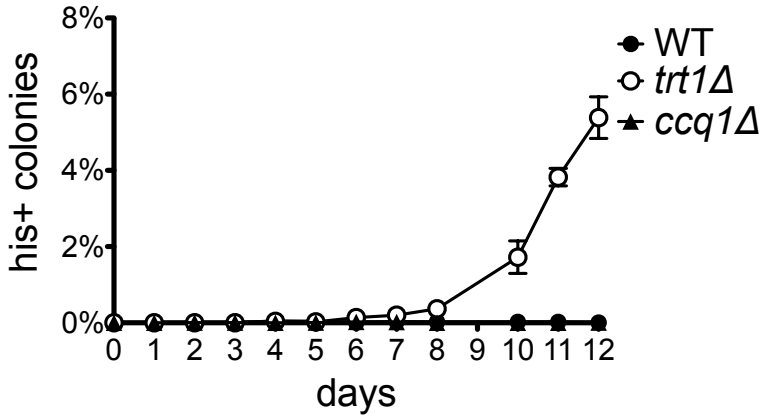


Figure 2.12. Frequency of *ccq1Δ* strains able to express histidine. Samples retrieved from liquid cultures were plated in solid media. Percentages were calculated by dividing the number of colonies growing in +HIS -LEU and -HIS -LEU. Error bars represent SEM of 3 replicates for each time point.

Thus, the pFT2 plasmid was unable to detect fusions in strains with recombinogenic telomeres. However, it detected fusions after gradual telomere erosion, in a quantitative manner. This property encouraged further investigation of the outcomes of telomere dysfunction in the context of absence of telomerase.

2.3 - Telomere length homeostasis in a strain containing extrachromosomal telomeres

2.3.1 - General aim

Previous work by Runge and Zakian using linear plasmids showed that the addition of extrachromosomal telomere sequences to budding yeast cells increases the overall set-point telomere length (Runge and Zakian, 1989). Telomere elongation occurs equally after the introduction of internal or terminal telomeres tracts, and is proportional to plasmid copy number.

Preliminary data from the initial characterization of pFT2 suggested that linear plasmids in fission yeast could affect telomere length in a similar way. Southern blot analysis of WT cells carrying pFT2 revealed that a slight upwards shift in the size of telomere fragments corresponding to endogenous telomeres, when compared to the control sample (Figure 2.5). A similar shift was observed for the ends of pFT2, which produced longer DNA fragments than what was predicted from its original sequence. The *his3*$^{+}$ promoter-proximal plasmid end was represented by a diffuse band of telomere tracts above 2.5 Kb, longer than the expected 2 Kb. Similarly, the telomere band corresponding to terminator-proximal plasmid end sat above the expected 1 Kb (Figures 2.2 and 2.13).

I proceeded to characterize the extent to which pFT2 altered telomere length. As observed for *taz1Δ* and *rap1Δ* mutants (Ferreira and Cooper, 2001; Miller et al., 2005), effects that deregulate telomere elongation could also be associated with telomere deprotection, which could confound the interpretation of the results.

There are several previous indications that the set-point for telomere length is influenced by limiting concentrations of specific telomere

components. In human cells, telomerase is a limiting factor for telomere elongation, and overexpression of both its catalytic and RNA components result in an increase in telomerase activity and telomere size (Cristofari and Lingner, 2006). Similarly, overexpression of hTPP1 leads to an increase in telomerase activity and telomere elongation (Nandakumar et al., 2012). The dose-dependent nature of telomere elongation is also illustrated by the study of haploinsufficiencies. In mice, heterozygous mutants for mTERT and mTR show telomere shortening and telomere elongation defects, respectively (Hathcock et al., 2002; Liu et al., 2000). Human cells heterozygous for hKU86 similarly show telomere shortening and instability (Myung et al., 2004). In budding yeast, heterozygous mutants for the RNA component of telomerase, Sc*TLC1*, have shorter telomeres (Mozdy, 2006; Mozdy et al., 2008). In addition, combinations of heterozygous mutations for different *est* genes result in early senescence for the mutant haploid offspring, a phenomenon dubbed *additive haploinsufficiency* (Lendvay et al., 1996).

In contrast, telomere elongation due to insufficient dosage of telomere components, has been alluded by indirect evidence. The addition of extrachromosomal telomeres to budding yeast mimics the phenotypes of a ScRap1 truncation which does not bind to DNA, ScRap1ΔBB (Wiley and Zakian, 1995). These phenotypes, which include telomere elongation and loss of TPE, suggest that ScRap1 is normally present in limiting amounts and is therefore unable to cope with significant increases in the number of telomeres. In fission yeast, overexpression of Taz1 in *rap1Δ* cells results in a reduction of the telomere elongation phenotype (Dehé et al., 2012). Thus, it is likely that WT concentrations of Taz1 are insufficient to cover the whole extent of *rap1Δ*'s elongated telomeres.

Given this, I proceeded to investigate whether telomere elongation after pFT2 transformation was a consequence of limiting expression of Shelterin components.

2.3.2 - Increasing the number of telomeres leads to overall telomere elongation

I conducted Southern blot analyses to assess telomere length after transforming pFT2 into fission yeast cells. Genomic DNA was digested with the ApaI restriction enzyme. This restriction increases the resolution of telomere length analysis, but does not distinguish chromosomal telomeres from plasmid telomeres (Figure 2.13).

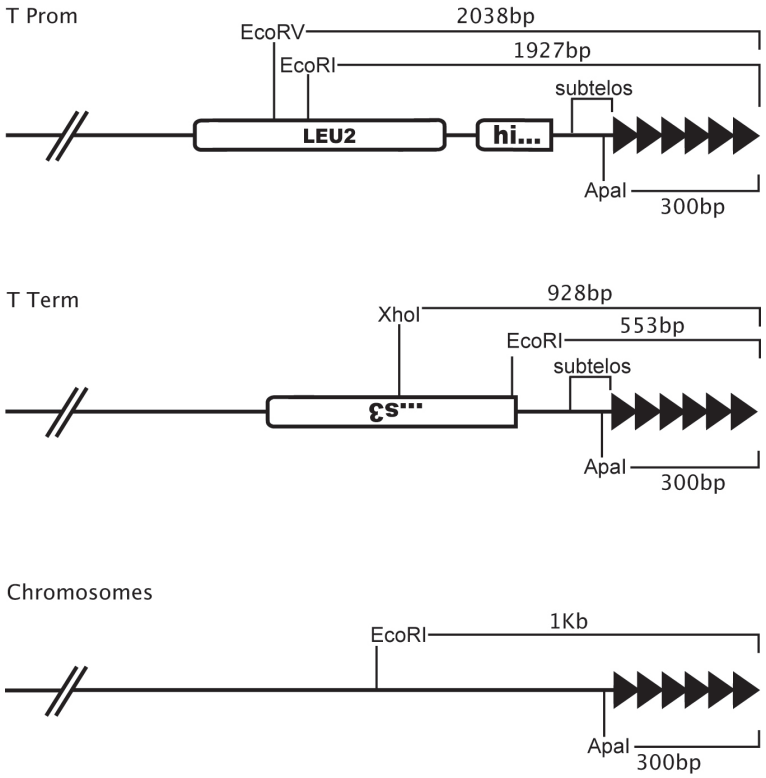
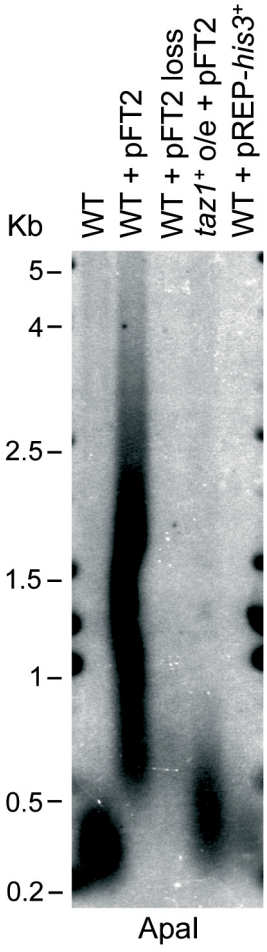


Figure 2.13. Detailed diagram representing enzyme restriction sites used for Southern Blot analysis of telomere sizes at T Prom, T Term, and chromosome ends. Black arrows indicate telomeres and white boxes indicate coding sequences. "Subtelos" indicate the subtelomeric sequences preceding the telomeres. The expected size of each digested fragment is indicated, for each restriction site.

Compared with the control, the strain carrying pFT2 had 3- to 5-fold elongated telomeres, and a much broader range of telomere sizes (Figure 2.14, lanes 1 and 2). This effect was not observed when cells were transformed with a control plasmid (Figure 2.14, lane 5).

Figure 2.14. Introducing extrachromosomal telomeres in fission yeast cells leads to overall telomere elongation. Southern blot analysis of telomere length in WT strains carrying pFT2, after pFT2 loss and with taz1^{+} o/e. Genomic DNA extracts were digested with ApaI and probed for telomere DNA. ApaI digestion does not discriminate between endogenous and pFT2 telomere ends.



To establish if the telomere elongation phenotype was exclusive to plasmid telomeres, I digested the genomic samples with the EcoRI enzyme. This restriction is able to separate telomeres originated in the chromosomes from the telomeres at each of pFT2's ends (Figure 2.13). Southern blot analysis confirmed that presence of the pFT2 plasmid also elongated the endogenous telomeres (Figure 2.15, lanes 1 and 2).

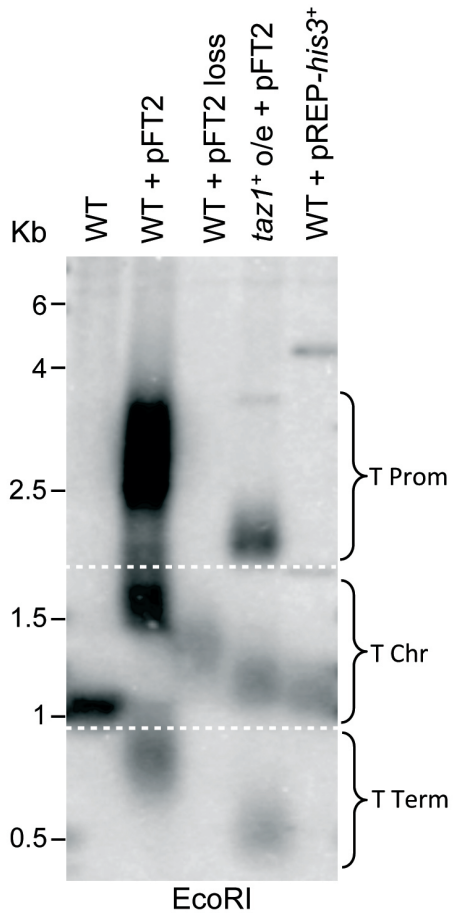


Figure 2.15. Introducing extrachromosomal telomeres in fission yeast cells leads to telomere elongation at chromosomes. The same genomic DNA extracts analyzed in figure 2.12 were digested with EcoRI and probed for telomere DNA. EcoRI digests separate the chromosomal telomeres (≈1 Kb) from the mini-chromosome telomeres.

This effect does not require terminal telomere sequences, as introducing pFT2 in the circular configuration also results in telomere elongation (Figure 2.16).

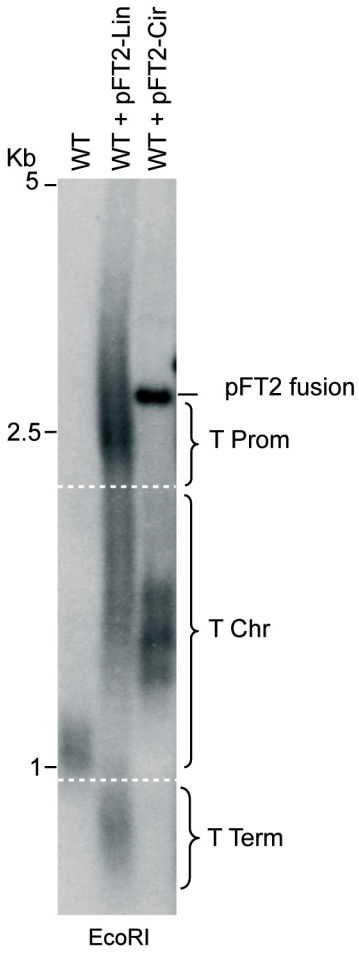
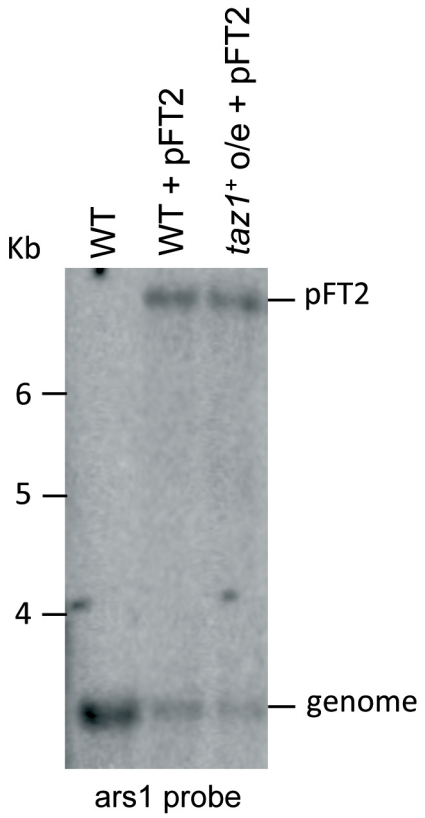


Figure 2.16. Circular plasmids with internal telomere tracts increase telomere length in chromosomes. Southern blot analysis of telomere length in WT strains carrying pFT2 in the linear (pFT2-Lin) and the circular (pFT2-Cir) conformations. Genomic DNA extracts were digested with EcoRI and probed for telomere DNA.

In budding yeast, changes in telomere length caused by linear plasmids are reversible and dependent on the continuous presence of the linear plasmids (Runge and Zakian, 1989). This was also the case for pFT2, since telomeres shortened in cells that were allowed to lose the linear plasmid (Figures 2.14 and 2.15, lane 3).

Haploid fission yeast cells spend most of their cell cycle in G2 phase, when they possess 2 sister chromatids for each of their 3 chromosomes. Thus, they normally carry a maximum of 12 telomeres during their cell cycle. To better assess the effect of adding extrachromosomal telomeres to WT cells, I determined how many pFT2 plasmids were present on average, per cell. Southern blot analysis was used to quantify the intensity of *ars1* signal, which is present as a single copy both in the genome and in pFT2 (Figure 2.17). Given that natural plasmid loss causes a fraction of the cells to lose the plasmid, even when under selection, I calculated that the plasmid is in the range of 5-7 copies per cell. Thus, introducing pFT2 in WT cells doubles the number of telomeres normally present at any given time.

Figure 2.17. Southern blot analysis of ars1 sequence in cells bearing the pFT2 mini-chromosome. Genomic DNA extracts were digested with NsiI enzyme and probed for ars1. Average number of pFT2 mini-chromosomes per cell was assessed by dividing the signal intensities of the ars1 sequence present in the mini-chromosome (top band) with the signal intensity of endogenous *ars1* (bottom band). Average number of mini-chromosomes per cell was finally calculated assuming that only 30% of the cells in a population maintain the mini-chromosome, at any given moment (Figure 2.6)



2.3.3 - Taz1 is the limiting factor in telomere length regulation

The counting mechanism model proposed for telomere length regulation predicts an increase in telomere size when there is a decrease in binding of telomere length regulators (Levy and Blackburn, 2004; Marcand et al., 1997; Miller et al., 2005; Wotton and Shore, 1997). Thus, it was possible that pFT2 was diluting a telomere length regulator present in limiting quantities.

If there was indeed a limiting factor for telomere length regulation, it was likely that overexpression of this component would compensate the elongation phenotype. To test this hypothesis, we overexpressed 4 well characterized Shelterin components in a plasmid with a promotor of moderate strength – Taz1, Rap1, Pot1 and Ccq1. Each candidate was chosen for its role in telomerase recruitment and regulation (Bunch et al., 2005; Miller et al., 2005; Miyoshi et al., 2008; Moser et al., 2011; Pitt and Cooper, 2010; Tomita and Cooper, 2008; Yamazaki et al., 2012).

Strains carrying pFT2 and overexpressing each of the candidate genes were propagated for 7 days in liquid culture, to allow for telomere shortening. Samples from these cultures were taken on the first and last days of the experiment. ApaI digests were performed for each sample, and probed for telomere repeats in a Southern blot analysis (Figure 2.18). A control plasmid had no effect in telomere length (Figure 2.18, lanes 12 and 13). However, overexpression of Taz1 had a dramatic effect and resected telomeres down to WT size (Figure 2.18, lanes 4 and 5). Although no changes in telomere length were seen with the overexpression of Rap1 and Ccq1, both strains seemed to have unstable telomeres. In addition to the expected telomeric smears, each strain presented several defined telomere bands of unknown origin (Figure 2.18, lanes 6, 7, 10 and 11). These are usually caused by telomere rearrangements and fusions (Pobiega and

Marcand, 2010). Interestingly, a similar result was observed in budding yeast where, although over-expression of ScRap1 results in telomere elongation, it also leads to chromosome instability (Conrad et al., 1990). Overexpression of Pot1 leads to a decrease in telomere size throughout the experiment (Figure 2.18, lanes 8 and 9). In fact, previous experiments showed that reducing Pot1 binding at telomeres results in telomere elongation (Bunch et al., 2005). Thus, Taz1 is normally present in limiting quantities, and its concentration is determining for the set-point of telomere length.

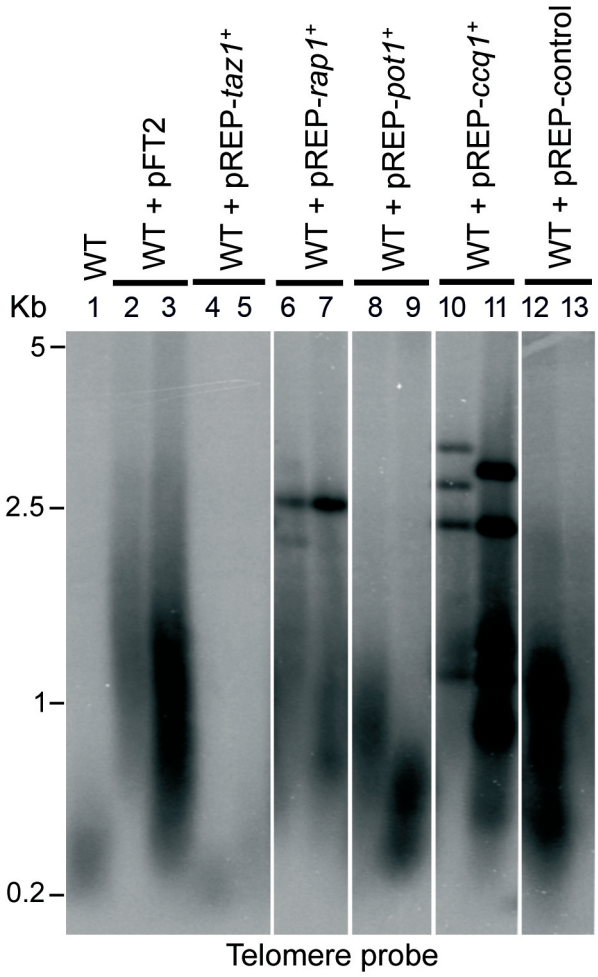


Figure 2.18. Linear pFT2 plasmid affects telomere length through dilution of Taz1.
Southern blot analysis of telomere length in WT strains carrying pFT2, after pFT2 loss and with taz1^{+} o/e. Genomic DNA extracts were digested with ApaI and probed for telomere DNA. ApaI digestion does not discriminate between endogenous and pFT2 telomere ends.

As dilution of Taz1 resulted in moderate elongation of telomeres, it was possible that strains carrying pFT2 had additional *taz1Δ*-like phenotypes. As described previously, I was unable to detect an increase in histidine-producing colonies upon a G_1-arrest, in these cells. Given this, I additionally tested for cold sensitivity in this strain, as it is also a *taz1Δ*-associated phenotype (Miller et al., 2006). WT cells carrying pFT2 had reduced viability at 20ºC, when compared to growth at 32ºC (Figure 2.19). This suggests that, like in *taz1Δ* strains, telomeres from WT strains carrying pFT2 are subjected to replication fork stalling. As expected, overexpression of Taz1 compensated for this phenotype (Figure 2.19).

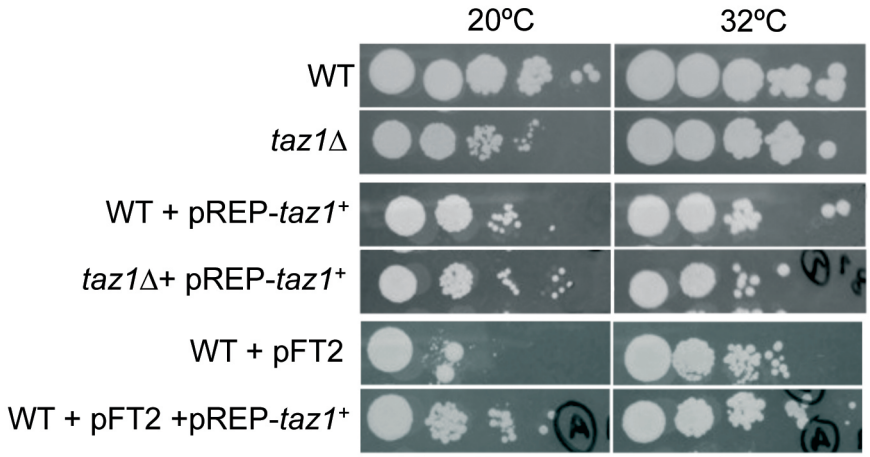


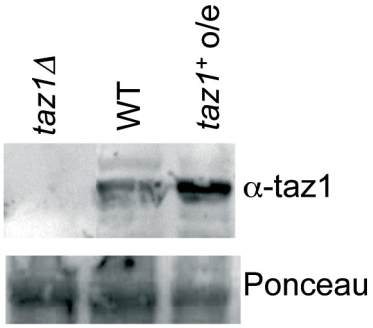
Figure 2.19. Conditions of Taz1 deficiency lead to cold sensitivity. Tenfold serial dilutions of each strain were plated in +HIS -LEU media and incubated at 20ºC or 32ºC

To construct a model that would resemble WT cells, I integrated a Taz1-overexpression cassette into a strain by inserting the pREP-taz1+ construct at the *taz1*$^+$ locus (see *Materials and Methods*, section 2.1.3). The construct was inserted by using the *taz1*$^+$ sequence for recombination. The strain, which bears both a WT *taz1*$^+$ copy, and a *taz1*$^+$ copy under the nmt1

promoter, was named *taz1*$^{+}$ *o/e*. It has been suggested that plasmid DNA competes for cellular maintenance (Eberhard, 1980). Additionally, selective pressures could cause a decrease in the average number of pFT2 copies per cell. Furthermore, fluctuations in Taz1 expression due to natural plasmid loss could result in a heterogenous population. The effect of such heterogeneity could have unforeseen outcomes for telomere protection and length regulation and confound the interpretation of our results We therefore predicted that integrating Taz1 overexpression would avoid confounding effects from the use of a second plasmid.

Integrated overexpression of Taz1 was assessed by Western blot, which indicated a two-fold increase in comparison to WT levels (Figure 2.20). Concomitantly, telomeres in the *taz1*$^{+}$ *o/e* strain carrying pFT2 were shortened almost to WT length (Figures 2.14 and 2.15).

Figure 2.20. Endogenous Taz1 overexpression. Overexpression of Taz1 was monitored on a western blot using α-taz1 (top) and Ponceau staining as loading control (bottom).



In conclusion, the number of telomeres present in fission yeast greatly influences telomere length homeostasis. We show that this is because Taz1 is usually present in limiting quantities. Even though it is not the sole known telomerase inhibitor (Miller et al., 2005, its overexpression is sufficient to compensate the telomere elongation phenotype observed in cells carrying extrachromosomal telomeres.

2.4 - Mechanisms of DNA repair at gradually eroding telomeres

2.4.1 - General aim

The ability to capture telomere fusions can be used to investigate the mechanism and outcomes of these end-joining reactions. These studies can help elucidate chromosome end-joining events in higher eukaryotes, where they have been widely recognized as a source of instability that precedes cancer (Artandi et al., 2000; Shay and Wright, 2011).

The mechanisms of fusion to which telomeres are subjected depend on the kind of dysfunction that renders telomeres unprotected. In *S. pombe*, telomere-to-telomere fusions in *taz1Δ* cells are NHEJ-mediated events (Ferreira and Cooper, 2001). Human TRF2 mutant cells are also subjected to telomere-to-telomere NHEJ-mediated fusions (Sarthy et al., 2009; Smogorzewska et al., 2002; Van Steensel et al., 1998). In contrast, extensive degradation of chromosome ends in *pot1Δ* and *trt1Δ* cells lead to SSA-mediated fusion reactions (Wang and Baumann, 2008). Similarly, in mice, *C. elegans* and Arabidopsis, telomerase deficiency results in chromosome end-fusions that are independent of NHEJ (Heacock et al., 2004; Heacock et al., 2007; Lowden et al., 2008; Maser et al., 2007; Riha and Shippen, 2003). In Arabidopsis, these fusions are mediated by stretches of microhomology and can, in some cases, involve telomere sequences or complex rearrangements (Heacock et al., 2004). In human cells, bypassing senescence results in fusions between critically short telomeres, which also involve regions of microhomology (Capper et al., 2007).

2.4.2 - *trt1*Δ extrachromosomal telomere fusions are mediated through the Single Strand Annealing pathway

Thus, NHEJ and SSA are the likelier culprits of the fusions detected with pFT2. Moreover, the outcome of these fusions could be diverse, involving telomeres, subtelomeric sequences and complex rearrangements. To assess the role of NHEJ and SSA in pFT2 end-fusions we generated *taz1*$^{+}$*o/e trt1*Δ strains carrying the pFT2 plasmid. These strains were further engineered to be defective for *lig4*$^{+}$ or *rad16*$^{+}$, essential components of the NHEJ and SSA pathways. Liquid cultures of each strain were followed for 18 days to allow for telomere erosion. Samples from these cultures were harvested every two days and plated in solid media, in the presence or absence of histidine, to assess the frequency of end-joining reactions. Southern blot analysis shows that telomeres in early generation *taz1*$^{+}$*o/e trt1*Δ are already much shorter than what was previously observed for the *trt1*Δ mutant (Figures 2.10 and 2.21).

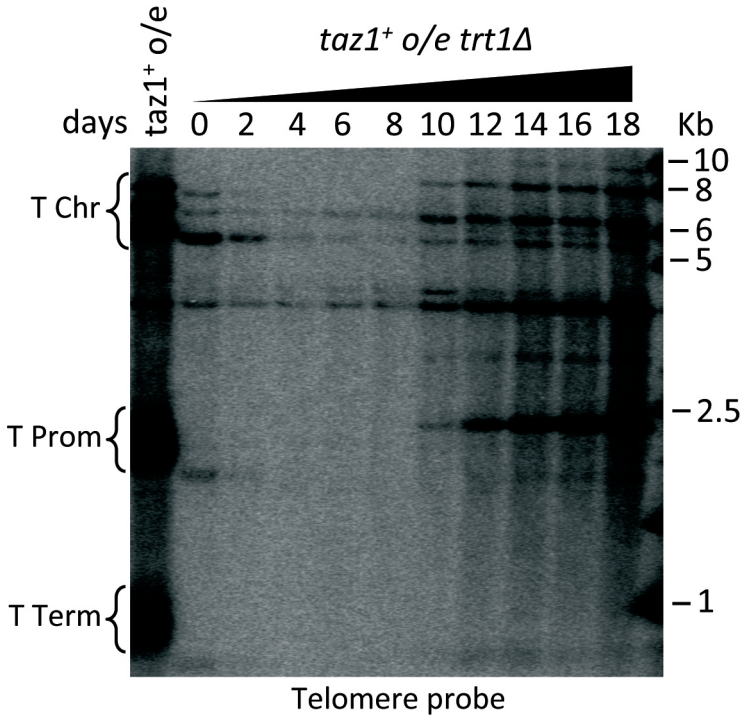


Figure 2.21. Southern blot analysis of telomere shortening in a *taz1*$^{+}$ *o/e trt1Δ* strain. Cells were grown in +HIS -LEU liquid media for 18 days and samples were retrieved every 2 days for Southern blot analysis. Genomic DNA extracts were digested with EcoRV and EcoRI. Promoter-proximal (T Prom), terminator-proximal telomeres (T Term) and chromosomal telomeres (T Chr) are depicted.

In fact, by day 4, pFT2 telomeres are completely undetectable. This is attributable to the shorter telomeres present in the *taz1*$^{+}$*o/e* strain, when compared to the WT strain carrying pFT2.

taz1^{+}*o/e trt1Δ* and *taz1*$^{+}$*o/e trt1Δ lig4Δ* strains were able to produce an increasing amount of histidine-producing colonies (Figure 2.22). This is consistent with previous observations that demonstrate that Lig4 is not required for the generation of telomerase mutant survivors (Baumann and Cech, 2000). However, histidine-producing colonies were almost absent in the *taz1*$^{+}$*o/e trt1Δ rad16Δ* mutant. These results are in agreement with previous reports that Rad16 is required for chromosome-end fusions in the absence of telomerase (Wang and Baumann, 2008).

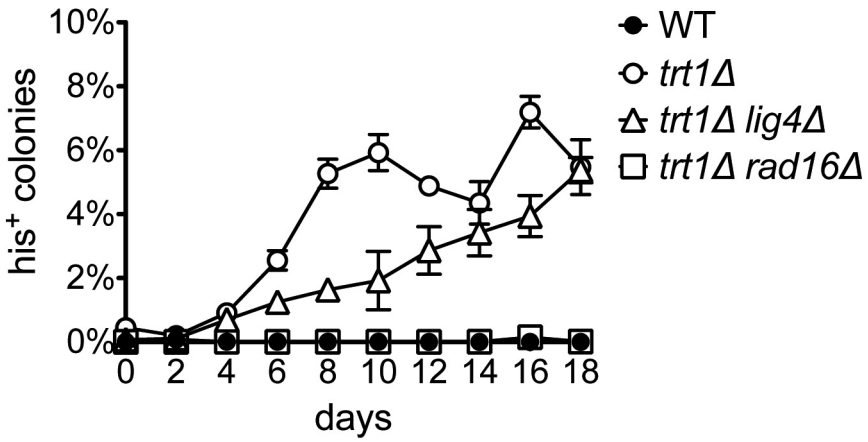


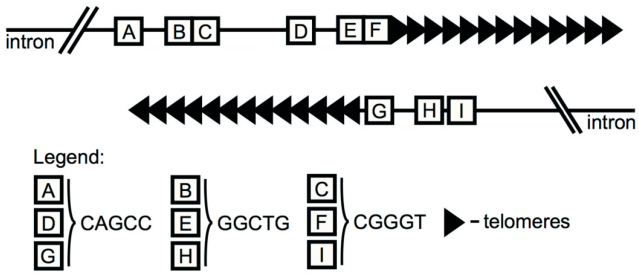
Figure 2.22. pFT2 fusions in *trt1Δ* occur via *rad16*-dependent SSA/MMEJ repair. Expression of histidine during telomere erosion in *trt1Δ, trt1Δ lig4Δ* and trt1*Δ rad16Δ* strains. Cells were grown in +HIS -LEU liquid media for 18 days. Samples were retrieved from each culture every 2 days and plated in solid +HIS -LEU and -HIS -LEU media. Frequencies were calculated by dividing the number of colonies growing in +HIS -LEU and -HIS -LEU. Error bars represent SEM of 3 replicates for each time point.

Thus, our work shows that in the absence of telomerase, the SSA pathway is required for pFT2 plasmid fusions. Furthermore, NHEJ is dispensable for these events.

2.4.3 - End fusions in *trt1*∆ mutants are mediated by specific sequences of microhomology present at the subtelomeres

In *pot1Δ* cells, where telomere resection is immediate and rampant, SSA mediates fusions only after several kilobases of chromosome ends have eroded (Wang and Baumann, 2008). In this model, there are microhomologies at the fusion junctions that can measure up to several hundred base pairs. I investigated whether SSA-mediated fusions in the pFT2 plasmid had a similar outcome.

For that end, I sequenced the fusion junctions in histidine-producing colonies of *taz1⁺o/e trt1Δ* and *taz1⁺o/e trt1Δlig4Δ* mutants. Primers were designed outside the intron sequence for PCR amplification. All analyzed plasmids retained an intact intron sequence. Consistent to what was previously published, only one sample retained telomere sequences (Hackett et al., 2001; Hemann et al., 2001; Nakamura et al., 1998). The large majority of fusions (93% in *trt1Δ* and 100% in *trt1Δlig4Δ* samples) occurred within the 85 bp of subtelomeric sequences cloned in the plasmid (Figure 2.23).



taz1⁺ o/e

fusion type	*trt1Δ*	*trt1Δlig4Δ*
A B C I	48.9%	27.3%
A B H I	15.6%	15.9%
A G H I	0%	0%
D E F I	24.4%	43.2%
D E H I	0%	11.4%
D G H I	4.4%	2.3%
Other	6.7%	0%

Figure 2.23. SSA-dependent fusions in *taz1⁺ o/e trt1Δ* cells are mediated by specific 5 bp microhomologies. Above: schematic representation of the 5nt microhomologies found at fusion junctions in *trt1Δ* and *trt1Δ lig4Δ* strains. Below: frequency of each microhomology at fusion junctions. *trt1Δ* strain, n=45; *trt1Δ lig4Δ* strain, n=44.

Surprisingly, three G-rich pentanucletide sequences were systematically present at the junction of these fusions. These pentanucleotides are normally found in chromosomal subtelomeric sequences (Sugawara, 1989). In the cloned telomeric and subtelomeric sequences of pNSU70 (Sugawara, 1989), there are 3 CGGGT microhomologies present within a single subtelomeric sequence, and one additional inverted copy. Likewise, the CAGCC microhomolgoy can be found twice, while its complementary sequence GGCTG is found 3 times. The GGCTG sequence is always found close to the CGGGT microhomology as GGCTGACGGGT, as observed for the pFT2 plasmid. However, with the exception of the sequence cloned in the pFT2 plasmid, the GGCTGACGGGT sequence is separated from the CAGCC sequence by several hundreds of base pairs (the closest distance being 2056 bp). In the pFT2 plasmid, they are present as 30 bp modules of direct repeats at the ends of pFT2, two promoter proximal (depicted as ABC and DEF) and one terminator proximal (GHI; Figures 2.23 and Appendices A and B). Since there were other microhomologies present within the cloned subtelomeric sequences, such as the ApaI restriction site, it was somewhat perplexing that there was a preference for these specific microhomologies. Furthermore, an additional 6 bp microhomology, TCTAGA, is found immediately adjacent to the subtelomeric sequences, which was not found at the fusion junction of any analyzed fusions (Appendix B). In addition, the pentanucleotide present in the CFI group was the most represented at fusion junctions (~70% in both *trt1Δ* and *trt1Δlig4Δ* strains, Figure 2.23). It became apparent that the remaining pair ADG/BEH was a palindrome separated by 14 bp that could form a hairpin structure (Figure 2.24). As the 5'-strand is degraded during telomere erosion, these sequences could become exposed and form secondary structures. These structures could

stabilize the ssDNA and promote annealing reactions at the adjacent pentanucleotide.

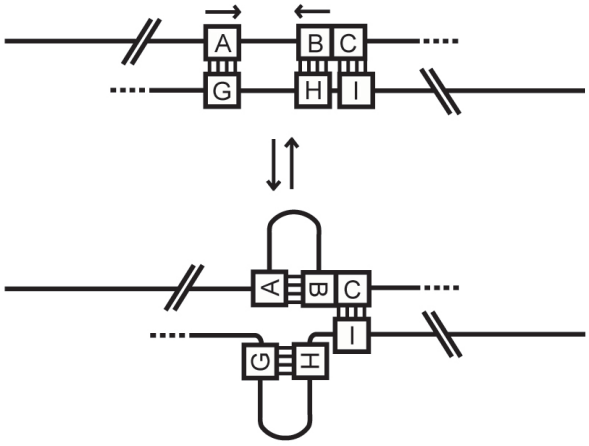


Figure 2.24. Hairpin model for microhomology-mediated fusions in *trt1Δ* and *trt1Δ lig4Δ* strains.

In other organisms, fusions are often detected between very short telomeres, after telomere erosion (Capper et al., 2007; Heacock et al., 2004). Thus, telomeres have a minimal functional length, from which point on they no longer guarantee chromosome-end protection (Abdallah et al., 2009; Capper et al., 2007). Therefore, pFT2 should be able to capture fusions with telomeres at their junctions. It was intriguing to find that, with the exception of one case, where a very short telomere fused to a subtelomeric end, none of the fusion junctions in the *taz1*$^{+}$*o/e trt1Δ* and *taz1*$^{+}$*o/e trt1Δlig4Δ* had telomere sequences (Figure 2.23, Appendix B). To test whether this could have been influenced by the overexpression of Taz1, a *trt1Δ* mutant with no integrated overexpression of Taz1 was allowed to undergo telomere erosion until histidine-producing colonies arose. Sequencing analysis of these fusion events revealed a very similar

pattern of plasmid-end fusions, confirming that this result was not specific of the *taz1*$^{+}$*o/e* strain (Figure 2.25).

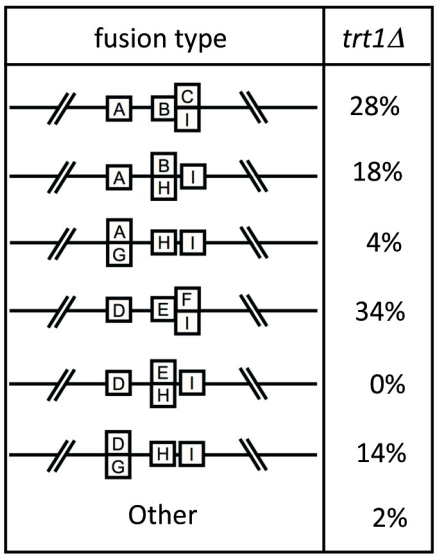


Figure 2.25. Fusions in *trt1Δ* cells, in conditions of Taz1 deficiency, are mediated by 5 bp microhomologies.

2.4.4 - The MRN complex is required for pFT2-end fusions in *trt1*Δ

If hairpin structures were involved in *trt1Δ* fusions, then removing hairpin-processing components from fission yeast, such as MRN or Ctp1, should have one of two outcomes: 1) inhibit the appearance of histidine-producing colonies, or 2) change the pattern of microhomologies found at fusion junctions.

To test this hypothesis, *taz1*$^{+}$*o/e trt1Δ* strains were constructed that lacked the three components of MRN and *ctp1*$^{+}$. These were allowed to undergo telomere erosion and were plated in the presence and absence of histidine to quantify the frequency of histidine-producing fusions. Absence of MRN or

Ctp1 impaired the *trt1Δ* survivor colonies from producing histidine (Figure 2.26).

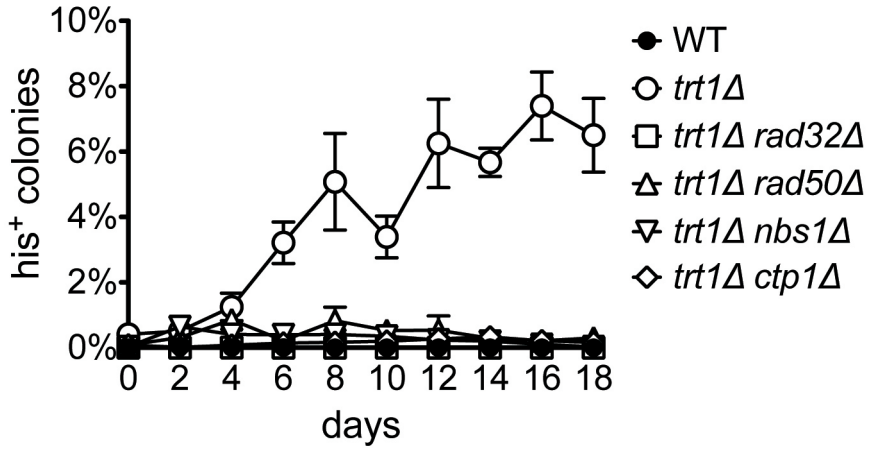


Figure 2.26. pFT2 fusions in *trt1*Δ require MRN/Ctp1. Expression of histidine during telomere erosion in *trt1Δ*, *trt1Δ MRNΔ* and *trt1Δ ctp1Δ* strains. Cells were plated daily on +HIS -LEU and -HIS -LEU media. Frequencies for were calculated by dividing the number of colonies growing in +HIS -LEU and -HIS -LEU. Error bars represent SEM of 3 replicates for each time point.

Thus, MRN and its partner Ctp1 are required for the microhomology-mediated fusions we observe in the pFT2 plasmid. Curiously, our findings are in accordance to what was reported in the *Arabidopsis* model, where telomere erosion leads to AtMre11-dependent microhomology-mediated chromosome-end fusions (Heacock et al., 2004). Furthermore, our work strongly supports the hypothesis that gradual erosion of telomeres leads to the formation of DNA hairpins, which require MRN and Ctp1 to be dismantled.

In conclusion, using pFT2, I quantitatively defined the progression of telomere fusions in *trt1Δ* mutants and other genetic backgrounds. The capture of single fusion events allowed for a qualitative evaluation of their

outcome. After undergoing telomere erosion, the ends of pFT2 fuse through the SSA pathway, in a MRN- and Ctp1-dependent manner. The existence of palindromic pentanucleotide microhomologies at fusion junctions suggests that hairpins define the progression of fusions in this background.

2.5 - Unperturbed fission yeast cells undergo spontaneous telomere-to-telomere fusions

2.5.1 - General aim

Previous reports had identified telomere fusions in telomerase-positive human cell lines. There are reports of microhomology-mediated fusions between critically short telomeres and subtelomeric sequences in immortalized epithelial cells (Capper et al., 2007). Additionally, critically short telomere-to-telomere fusions were detected in breast carcinoma cells (Tanaka et al., 2012). In both models, results suggest that fusions between telomere sequences occur specifically after critical telomere shortening, when telomere protection has been lost.

In WT budding yeast, DNA DSBs were also found to engage telomeres in fusion reactions (DuBois et al., 2002). In this system, telomeres of varying size were found at fusion junctions, suggesting that fully functional telomeres can become fusogenic.

Surprisingly, while characterizing the telomere fusion capturing assay, I noticed the appearance of rare histidine-producing colonies stemming from WT strains (Figure 2.3). These colonies could be continuously derived from strains carrying pFT2 in the linear conformation. These were initially unable to grow in the absence of histidine. This finding suggested that spontaneous telomere fusions could occur in unperturbed cells. Therefore, I pursued the investigation of the frequency and nature of

these fusions. Furthermore, I was interested to understand which repair mechanisms were involved in these spontaneous telomere fusions.

The find that unperturbed telomeres can participate in fusion reactions may contribute to the understanding carcinogenesis.

2.5.2 - Telomere fusions at unperturbed telomeres occur through the NHEJ pathway and require MRN/Ctp1.

Not all kinds of telomere dysfunction result from gradual telomere resection. Chromosome ends in *pot1Δ* mutants are subjected to rampant resection and are completely lost within 1 cell division, losing several Kbs off chromosome ends (Pitt and Cooper, 2010; Wang and Baumann, 2008). To test whether the spontaneous fusions found in WT cells would be exacerbated in conditions of from rampant resection of telomeres, I introduced pFT2 into a *pot1-1* ts mutant. This mutant has moderately elongated telomeres and is viable at 25ºC. However, at 36ºC Pot1 is no longer functional, resulting in immediate chromosome-end fusions (Pitt and Cooper, 2010). WT and *pot1-1* strains carrying pFT2 were plated and incubated at both 25 and 36ºC. Surprisingly, switching the *pot1-1* mutant to 36ºC did not result in the appearance of *his3*$^{+}$-producing colonies (Figure 2.27). It is possible that resection either destroyed the *his3*$^{+}$ coding region, or its ability to splice the intron properly, thus preventing the capturing of fusions. This could explain the absence of spontaneous fusions found in the WT strain (Figure 2.27). However, I did not assess this hypothesis by Southern blotting. Repeated experiments should reveal whether increasing temperature reduces the frequency of spontaneous fusions, as suggested by these results (Figure 2.27).

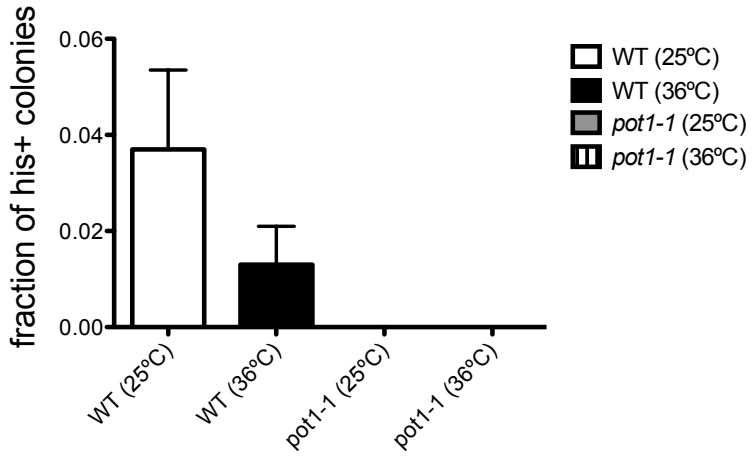


Figure 2.27. Absence of functional Pot1 dos increase pFT2 fusions. Frequency of fusions in *pot1-1* mutants at the permissive (25ºC) and restrictive (36ºC) temperatures. Cells were plated in +HIS -LEU and -HIS -LEU media in quadruplicate. Percentage of his+ cells in the population was determined by dividing the number of his+ colonies by the total number of colonies. Error bars represent standard error of 1 experiment. The threshold of detection is $1x10^{-5}$ his+ colonies/total sample.

I was initially concerned that the spontaneous fusions emerging in WT cultures were caused by telomere deprotection due to insufficient Taz1 levels. To determine the frequency at which these fusions occurred in the WT and in the *taz1*$^{+}$ *o/e* backgrounds, samples from liquid cultures of either strains were plated in media in the presence or absence of histidine. The frequency at which *his3*$^{+}$-expressing colonies appeared in the *taz1*$^{+}$ *o/e* strain was $1.4x10^{-4}$ ± $0.5x10^{-4}$ (SEM, n=3; Figure 2.28). In contrast, they appear at a frequency of $3.4x10^{-4}$ ± $0.9x10^{-4}$ (SEM, n=3) in a WT background, where Taz1 is limiting. Thus, limiting Taz1 quantities result in an increase in telomere length and a reduction in telomere protection, measured as an over 2-fold propensity to engage in fusions.

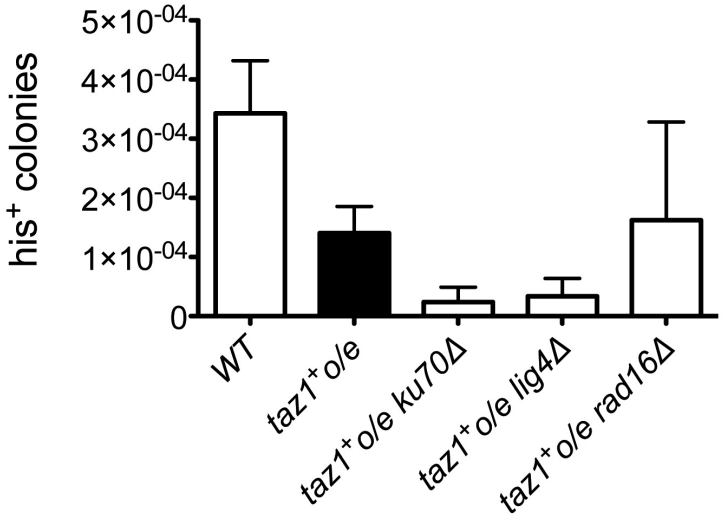


Figure 2.28. Spontaneous telomere fusions occur in unperturbed WT cells. Frequency of fusions in WT, and *taz1⁺o/e ku70Δ, taz1⁺o/e lig4Δ* and *taz1⁺o/e rad16Δ* strains. Cells were plated in +HIS -LEU and -HIS -LEU media in triplicate. Percentage of his+ cells in the population was determined by dividing the number of his+ colonies by the total number of colonies. Error bars represent SEM of 3 independent experiments. The threshold of detection is $1x10^{-5}$ his+ colonies/total sample.

To clarify the nature of these fusions, I examined their frequency in the absence of specific repair pathways, as it had been done for the *trt1Δ* fusions. For that end, *lig4⁺*, *ku70⁺* and *rad16⁺* were deleted from *taz1⁺ o/e* strains. Absence of the SSA component Rad16 did not disrupt the appearance of *his3⁺*-expressing colonies (Figure 2.28). However, absence of NHEJ, either through disruption of *lig4⁺* or *ku70⁺*, resulted in a dramatic reduction in the frequency of revertants (Figure 2.28). Thus, the NHEJ pathway mediates the spontaneous telomere fusions uncovered by the pFT2 plasmid. This suggests that these deprotection events are of a different nature than the ones found after gradual telomere erosion.

Even though the MRN complex is not required for NHEJ in fission yeast, as measured by plasmid repair assays (Manolis et al., 2001), a recent publication shows that NHEJ-mediated telomere fusions in *taz1Δ* strains require MRN (Reis et al., 2012). I assessed whether this requirement was recapitulated in the NHEJ-mediated telomere fusions of pFT2. Deletion of each of the MRN components inhibited the appearance of histidine-producing colonies (Figure 2.29). However, and in contrast to what was reported previously (Reis et al., 2012), Ctp1 was also required for these fusions. Therefore, spontaneous fusions resemble telomere-to-telomere fusions in G_1-arrested *taz1Δ* cells, in that they are NHEJ- and MRN-dependent. In addition, Ctp1 is also required for spontaneous telomere fusions.

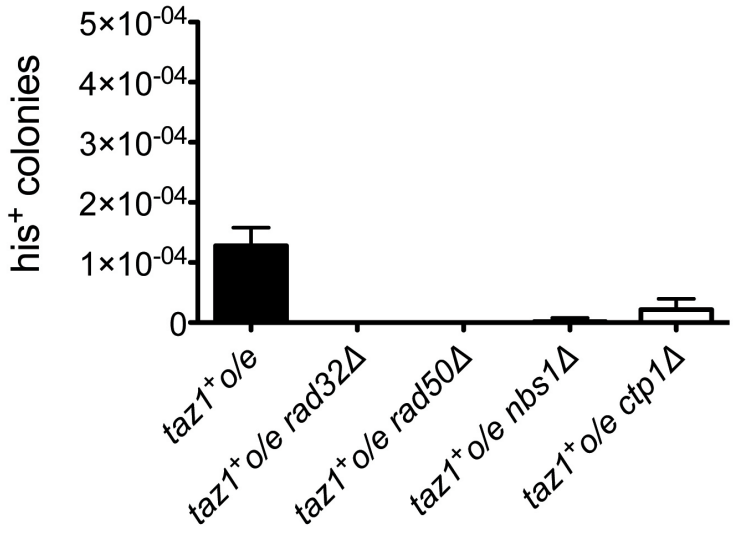


Figure 2.29. Spontaneous telomere fusions require MRN/Ctp1. Frequency of fusions in *taz1+o/e rad32Δ, taz1+o/e rad50Δ, taz1+o/e nabs1Δ,* and *taz1+o/e ctp1Δ* strains. Cells were plated in +HIS -LEU and -HIS -LEU media in triplicate. Percentage of his+ cells in the population was determined by dividing the number of his+ colonies by the total number of colonies. Error bars represent SEM of 3 independent experiments. The threshold of detection is $1x10^{-5}$ his+ colonies/total sample.

2.5.3 - Spontaneous telomere fusions and replication fork stalling

Due to their repetitive sequences, telomeres behave as fragile sites that are occasionally subjected to replication fork stalling and collapse (Gu et al., 2012; Leman et al., 2012; Miller et al., 2006; Sfeir et al., 2009). Therefore, the collapse of replication forks can compromise telomere protection and generate spontaneous telomeres fusions at unperturbed telomeres. If this were true, increasing the frequency of replication fork stalling events should increase the frequency of spontaneous telomere fusions. To address this hypothesis, *taz1*$^{+}$ *o/e* cells were subjected to a chronic Hydroxyurea treatment in solid media. Hydroxyurea is an inhibitor of the deoxyribonucleotide (dNTP) synthesizer Ribonucleotide reductase (Elford, 1968). This causes depletion of dNTPs, which leads to replication fork stalling. Additionally, they were grown at 20°C, which in *taz1*Δ mutants causes replication fork stalling at telomeres (Miller et al., 2006). To exclude general effects caused by DDRs, two control conditions were tested. In one, cells were plated in media containing Camptothecin, which is a drug that inhibits Topoisomerase I, thus generating DNA breaks (Wan et al., 1999). In another, cells were exposed to UV radiation, which causes thymidine bases to dimerize (Boddy and Russell, 2001). A *taz1*$^{+}$ *o/e his3*$^{+}$-producing revertant was added to test whether telomere fork stalling at fusion junctions would compromise the expression of *his3*$^{+}$. Expression of histidine was uninhibited in every condition (Figure 2.30). However, none resulted in a visible increase in spontaneous histidine-producing colonies (Figure 2.30). These results suggest that replication fork stalling at telomeres is not involved in generating spontaneous telomere fusions. However, chronic exposure to Hydroxyurea could compromise the stability of telomere fusions, thus preventing the expression of histidine. Further experiments with acute exposure to these drugs, followed with recovery in

solid media should clarify the role of replication fork stalling in spontaneous telomere fusions.

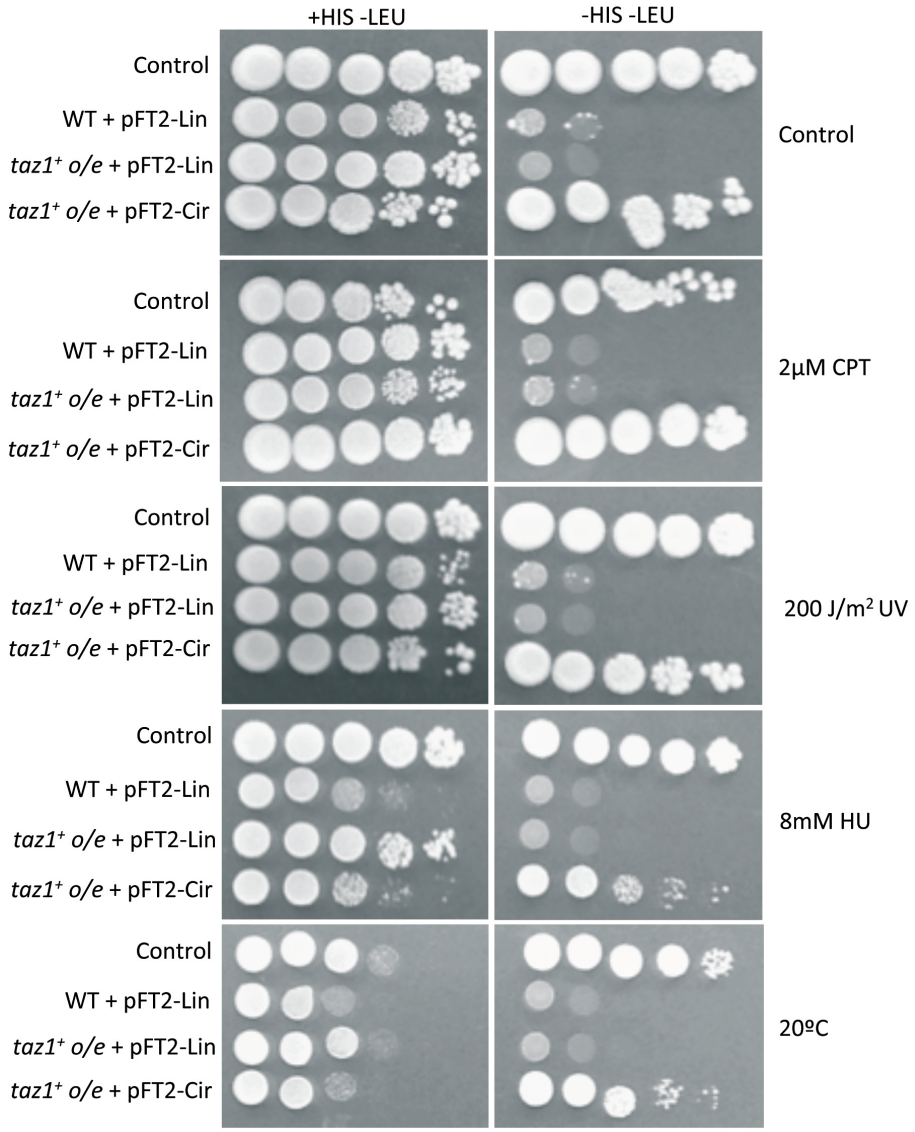
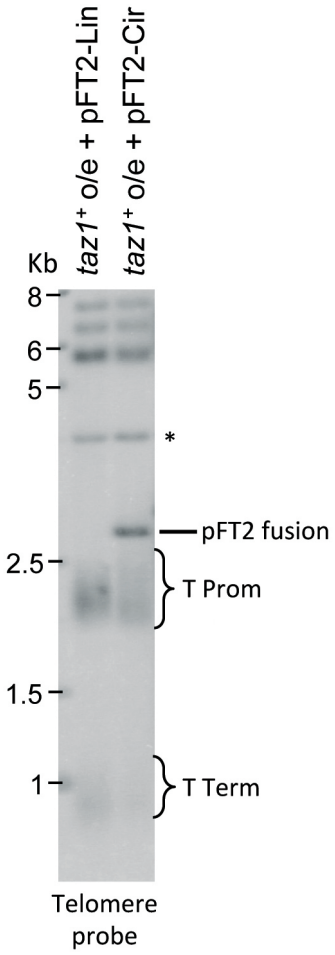


Figure 2.30. Spontaneous telomere fusions do not increase in conditions of chronic replication fork stalling. Tenfold serial dilutions of WT and *taz1*$^{+}$ *o/e* cells carrying either the linear (pFT2-Lin) or circular (pFT2-Cir) plasmid were plated on permissive (+HIS -LEU) or restrictive (-HIS -LEU) media, and subjected to different sources of DNA damage (CPT Camptothecin; UV – ultraviolet light; HU – Hydroxyurea). Control cells contain a *LEU2*-based plasmid that lacks the *his3*$^{+}$ gene.

2.5.4 - Characterization of fusions events at unperturbed telomeres

The requirement of NHEJ for the appearance of revertants in unperturbed cells suggested that they were driven by fusion events different than the ones observed in *trt1Δ* survivors. One prediction was that these fusions would not depend on homologies. Instead, Southern blot analysis demonstrated that telomere sequences were present at fusion junctions (Figure 2.31).

Figure 2.31. Spontaneous telomere fusions retain telomere sequences. Genomic DNA extracts were digested with EcoRV and/or XhoI enzymes and probed for telomeres (see Fig. 2.11 for details). Promoter-proximal (T Prom), terminator-proximal telomeres (T Term) and chromosomal telomeres (T Chr) are depicted. The asterisk (*) indicates unspecific labeling.



Amplification and sequencing analysis of the junctions of these fusions proved to be rather challenging. Telomeres are comprised of tandem direct repeats of a G-rich sequence, which makes them especially refractory to sequencing (Heacock et al., 2004; McEachern et al., 2000; Mieczkowski et al., 2003). I was able to sequence 28 fusion reactions. The predominant events found in this sample involved telomere tracts from each side of the fusion junctions (89.3%; Figures 2.32 and 2.33).

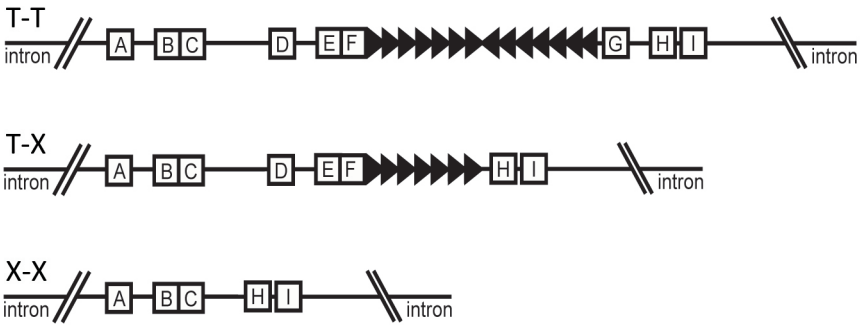


Figure 2.33. Three types of spontaneous telomere fusions. Classification of telomere fusion types. T-T (telomere to telomere): fusion junctions containing telomeric DNA from both ends. T-X: fusion junctions containing telomeric DNA on one end fused to a subtelomeric region. X-X: fusion junctions containing non-telomeric DNA.

A smaller percentage was comprised of telomere tracts fused with subtelomeric sequences (3.6%), or fusions between non-telomeric sequences (7.1%). No microhomologies were evident at fusion junctions. However, it cannot be ruled out that very small 1-2 bp microhomologies could be generated from G-rich sequences such as telomeres (Appendix C).

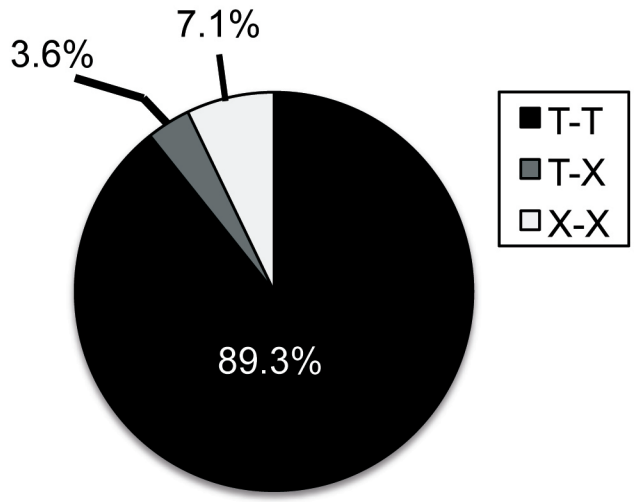


Figure 2.33. Telomere-to-telomere fusions are the main type of spontaneous telomere fusions. Frequency of T-T, T-X, and X-X fusions (n=28).

These results challenged previously held notions of telomere protection. Previous reports in immortalized human cells suggest that chromosome-end fusions only occur after loss of telomere protection by resection of telomeres (Capper et al., 2007; Tanaka et al., 2012). To understand if these fusions were the result of critical telomere shortening at one of the intervening ends, each telomere tract found at a fusion junction was organized in a length histogram (Figure 2.34). WT-length telomere tracts were frequent. However, this analysis revealed a 2-fold difference in median telomere size between the promoter proximal (T Prom 232; interquartile range (IQR) 92-291 bp), and the terminator proximal (T Term 111 bp; IQR 29-147 bp) telomeres of pFT2. Thus, on average, spontaneous telomere fusions occur between telomere tracts of about half the normal length observed in the linear configuration.

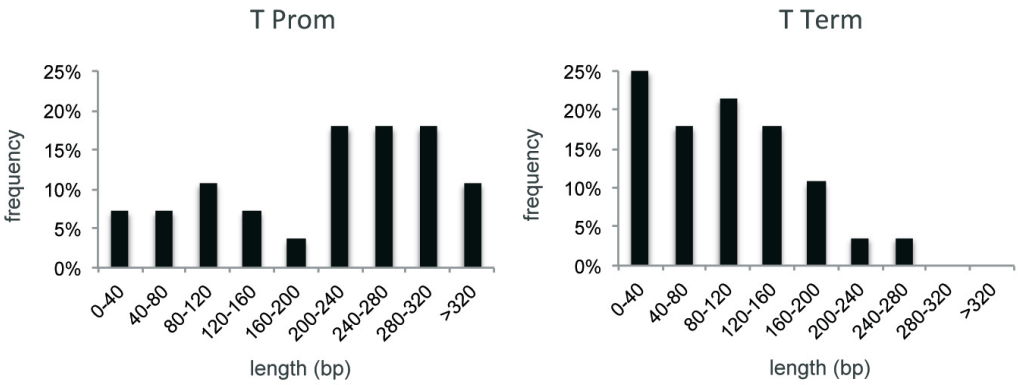


Figure 2.34. Mini-chromosomes undergo end fusions at half-sized telomere length. Frequency of telomere fusions according to telomere length, at the promoter-proximal telomere (T Prom) and at the terminator-proximal telomere (T Term).

As observed for DSBs in budding yeast (DuBois et al., 2002), it was possible that one critically short telomere would draw the opposing telomere into fusing, regardless of its size. However, the pattern of telomere fusions captured by pFT2 suggested otherwise. Telomere lengths at T Prom and T Term were positively correlated, and the longer the telomere was on one side, the longer it was on the other (R2 = 0.62, n = 28; Figure 2.35). Thus, even though there is a discrepancy in the telomere sizes of T Prom and T Term, these fusions cannot be solely caused by critical telomere shortening at one end of pFT2. It is, therefore, reasonable to suggest that these fusions are caused by momentary deprotection of otherwise functional telomeres.

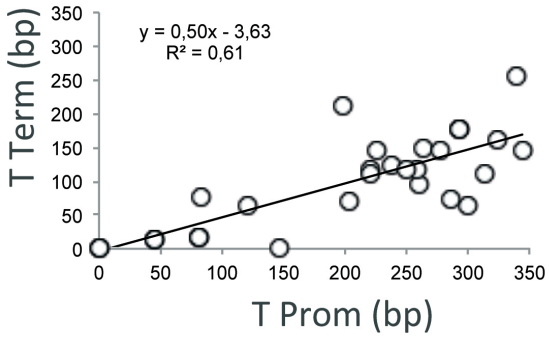


Figure 2.35. Terminator-proximal telomeres present at fusion junctions are approximately half the length of the opposite promoter-proximal telomere. Pairwise telomere length distribution of mini-chromosome telomeres involved in fusions.

T Term consistently participated in fusions at half the telomere size of T Prom. To understand if there was a size bias before the fusion events took place, I measured the lengths of both ends of pFT2 by Southern blot analysis (Figure 2.15, lane 4). For the *taz1*$^{+}$ *o/e* strain, average telomere lengths in the linear conformation of pFT2 revealed a 2-fold difference between T Prom and T Term (415 bp for T Prom and 184 bp for T Term). This seemed to be a general property of the pFT2 plasmid, as the plasmid retains a 2-fold difference between both ends even when it is propagated in conditions of Taz1 insufficiency (Figure 2.15, lane 2). Thus, the trend observed in telomere fusions could be justified by the telomere sizes normally present at T Prom and T Term.

The difference in length between both ends of pFT2 could be caused by increased activation or inhibition of telomerase at one side. T Prom along with the endogenous telomeres may be favorable telomerase substrates over T Term. This may be due to ongoing transcription of *his3*$^{+}$ or TERRA, which should be absent in T Term. If telomerase is more active at one end than the other, then its activity could be inferred by recognizable telomere addition events at the sequence level. For that end, I compared

the telomere sequences present at fusion junctions, with the telomere repeats originally present in the plasmid (Figure 2.36). As it was explained in section 1.3, fission yeast telomerase produces "errors" in the consensus sequence of telomeres, adding variably sized spacer sequences between the GGTTAC repeats (Leonardi et al., 2008; Webb and Zakian, 2012). These spacers would differentiate newly added sequences from the originally cloned repeats, and also reveal sites of telomere resection to which new sequences were added (Figure 2.36). Sequencing analysis showed that most of the T Prom telomeres that engaged in fusions were used as substrates by telomerase (26 samples out of 28; Figure 2.36). In contrast, little more than half of T Term telomeres showed signs of telomere repeat addition (16 out of 28; Figure 2.36). This result, along with the shorter telomeres observed in T Term, suggests that telomerase is more active at the T Prom telomere than at T Term. Once again, the size of original telomere sequences kept at fusion junctions showed a 2-fold difference between promoter and terminator proximal telomeres (T Prom 101 bp and T Term 68 bp; IQR 75-149 bp and 18-103 bp, respectively). These results suggest that T Term is elongated only half the times as the T Prom telomere, which causes it to possess shorter steady state telomere lengths.

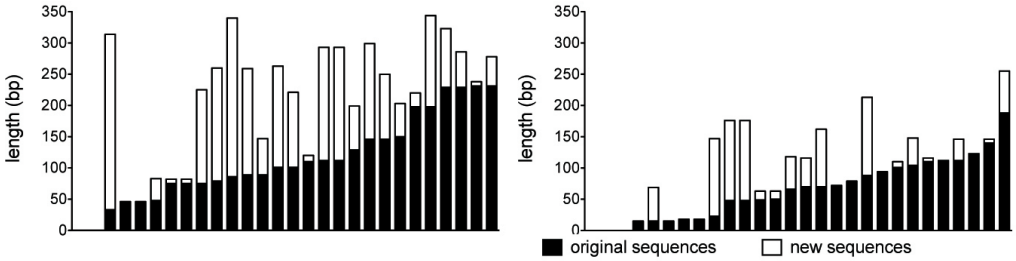


Figure 2.36. Telomere sequences present at fusion junctions reveal addition of new telomere repeats. Telomere size of each telomere (n=28). White bars represent originally cloned telomere sequences and black bars represent sequences added *in vivo*.

In conclusion, unperturbed cells are subject to spontaneous telomere-to-telomere fusions, mediated by the NHEJ pathway and MRN/Ctp1. These fusion events seem to be stochastic, and in average occur between telomeres at half their normal size.

Chapter 3 - Discussion

3.1 - Spontaneous telomere-to-telomere fusions have possible repercussions in our understanding of cancer and genome stability

The work here described introduces a novel method, which detects and captures single telomere fusion events. Using this method, based on a linear plasmid with telomeres at each end, I was able to quantify the frequency of fusions events and define the pathways and outcomes of fusion in several genetic backgrounds. Unexpectedly, this assay uncovered the occurrence of stochastic telomere-to-telomere fusions in unperturbed cells. Previously, critically short telomeres have been described to undergo fusions in telomerase-positive cell lines, while also being implicated in cancer (Artandi et al., 2000; Capper et al., 2007; Letsolo et al., 2010; Lin et al., 2010; Tanaka et al., 2012). However, this is the first time that fusions have been observed between unperturbed telomeres. These fusions were largely dependent on the NHEJ pathway, as was previously observed for telomere-to-telomere fusions in G_1-arrested *taz1*Δ cells, budding yeast Sc*rap1*Δ mutants and human hTRF2$^{F/-}$ mutants (Bae and Baumann, 2007; Ferreira and Cooper, 2001; Pardo and Marcand, 2005). In fission yeast, no fusions have been previously detected at *taz1*Δ telomeres of cycling cultures, which are subject to HR during S/G2 phase of the cell cycle (Ferreira and Cooper, 2001)(Rog et al., 2009). However, NHEJ-mediated fusions occur in cycling cultures if HR is compromised (Ferreira and Cooper, 2001).

Our data does not explain how the residual fusion events occur in the absence of NHEJ (Figure 2.28). However, it would be important to identify the backup pathway involved in these fusions. What we know already may provide some hints. In fission yeast, Ku effectively inhibits SSA-mediated repair (Decottignies, 2007). In budding yeast, a microhomology-mediated mechanism of end-joining has been identified in

the absence of ScKu (Yu and Gabriel, 2003). Concomitantly, mammalian NHEJ-deficient cells are able to religate DNA breaks through alt-NHEJ, a microhomology-mediated repair pathway (Fattah et al., 2010; Guirouilh-Barbat et al., 2004; Liang and Jasin, 1996; Simsek et al., 2011; Wang et al., 2003). This pathway has also been implicated in chromosome-end fusions following the complete removal of shelterin (Sfeir and de Lange, 2012). Thus, SSA is the likely backup pathway involved in the residual *lig4*$^{+}$- and *ku70*$^{+}$-independent fusions. If this is the case, then fusions should occur either via G-rich microhomologies at telomeres, or after their complete resection, as observed for *trt1*Δ mutants (Figure 2.25). Moreover, generation of histidine-producing colonies from unperturbed strains should be completely abolished in the absence of both NHEJ and SSA.

In accordance to what was previously published for chromosome-end fusions in *taz1*Δ cells (Reis et al., 2012), the MRN complex was required for the circularization of pFT2 in unperturbed cells (Figure 2.29). MRN may tether pFT2's telomeres together, as it was proposed for *taz1*Δ fusions. Although Ctp1 is not required for *taz1*Δ telomere fusions (Reis et al., 2012), the frequency of histidine-producing revertants drops considerably in *taz1*$^{+}$ *o/e ctp1*Δ mutants (Figure 2.29). Therefore, it is possible that Ctp1 has an additional role in processing telomere ends prior to fusion, as it is involved in 5'-strand resection of DSBs (Clerici et al., 2005; Limbo et al., 2007; Nicolette et al., 2010; Sartori et al., 2007; You et al., 2009). This function would not be required at *taz1*Δ telomeres, which possess overextended G-overhangs (Tomita et al., 2003).

The requirement of Ctp1, which is absent during G1 phase (Limbo et al., 2007), further suggests that these fusions occur outside of G1. It is therefore possible that the stochastic telomere fusions uncovered by the pFT2 plasmid are triggered by momentary deprotection during replication in late S phase. Telomeres are recognized as DNA damage during

replication, as part of a regular telomere maintenance program which includes the recruitment of many DDR components (Boulton and Jackson, 1998; Ferguson et al., 1991; Lustig and Petes, 1986; Metcalfe et al., 1996; Moser et al., 2011; Moser et al., 2009b; Nakamura et al., 2002; Verdun et al., 2005; Verdun and Karlseder, 2006; Wellinger et al., 1993a, b). Normally, telomeres are bound by these factors, but block the propagation of a DDR halfway in the signaling pathway (Carneiro et al., 2010; Okamoto et al., 2013; Verdun et al., 2005). Passage of the replication fork would open telomeres and expose chromosome-ends, thus permitting the engagement of telomerase, but also displacing the shelterin complex and cause momentary deprotection (Verdun et al., 2005; Verdun and Karlseder, 2006). Indeed, a model has been proposed for short telomeres, in which telomerase recruitment is dependent on transient recognition of telomeres as DSBs, through DDR components such as Tel1 and MRN (Bianchi and Shore, 2007b; Chang et al., 2007; Dehé et al., 2012; McGee et al., 2010; Sabourin et al., 2007; Verdun and Karlseder, 2006). The presence of DNA repair components may thus facilitate opportunistic telomere-to-telomere fusions as telomeres open to replication and telomerase recruitment. Additionally, Taz1 levels may be limiting during this period and fail to protect the newly duplicated telomeres. In support of this hypothesis, a strain with insufficient Taz1 undergoes telomere fusions at a higher frequency than a strain overexpressing Taz1 (Figure 2.28).

In general, telomere-to-telomere fusions may be an underestimated phenomenon, owing to the difficulties in quantifying and sequencing these events (Heacock et al., 2004; Lowden et al., 2008; McEachern et al., 2000; Mieczkowski et al., 2003). One assay quantified an extremely low frequency of fusion events between an artificially induced DSB and telomeres ($8.4x10^{-8}$; (DuBois et al., 2002). In contrast, telomere-to-telomere fusions in the pFT2 plasmid are more frequent by several orders of

magnitude ($1.4x10^{-4}$). This discrepancy may be explained by the differences in experimental setup. The telomere-to-DSB fusion assay created by DuBois and co-workers relies on coinciding a DSB with transient telomere deprotection. Asynchronous cultures of budding yeast are predominantly in G_1 phase, and induction and repair of a DSB may all occur before late S phase, when telomeres are replicated and possibly deprotected. In contrast, transient telomere deprotection during replication should be synchronous to all telomeres. This hypothesis may be addressed in the future by performing a telomere-to-DSB fusion assay, while controlling the cell cycle induction of the DSB via a G_1 arrest. Alternatively, the frequency of telomere fusions found through the assay described here may be a consequence of the artificial nature of the reporter construct. In contrast to endogenous chromosome ends, which possess subtelomeres that encompass 10 Kbp, the pFT2 plasmid has very short 80 bp subtelomeric regions. Testing the role of subtelomeres in telomere protection by increasing their size in the pFT2 plasmid should address this hypothesis. In addition, ongoing transcription from the *his3*$^+$ promoter may affect telomere protection. To address this issue, we may assess the frequency of telomere fusions after manipulating the expression of the marker gene through the use of promoters of different strength (Belén Moreno et al., 2000).

Replication fork stalling at telomeres could also be involved in the generation of fusions. Chronic exposure to Hydroxyurea, which causes replication fork stalling, did not cause an increase in telomere-to-telomere fusions (Figure 2.30). However, I did not assess whether acute exposure to hydroxyurea would increase the appearance of spontaneous telomere fusions. To further test whether replication fork stalling is a causative agent of telomere fusions, the involvement of Rqh1 should be assessed. Rqh1 is a helicase that causes telomere breakage and entanglement at *taz1*Δ

telomeres (Rog et al., 2009), an effect that is triggered by replication fork stalling (Miller et al., 2006). A mutant version of *rqh1*$^{+}$, *rqh1-SM*, supresses Rqh1's deleterious function in *taz1*Δ cells (Rog et al., 2009)). Thus, it could be tested whether this mutant is able to abolish the occurrence of histidine-producing escapers.

We also excluded spontaneous mutations in telomere components as the main source for the stochastic fusions we observe. Telomere fusions do not occur simultaneously in all pFT2 plasmids of a given cell. Southern blot analysis of recovered histidine-producing escapers shows that fused plasmids can coexist with linearized plasmids, even under selection for histidine production (Figure 2.31). Thus, theses fusions are unlikely to be caused by spontaneously arising constitutive telomere defects.

Finally, spontaneous fusions between otherwise functional telomeres may be an overlooked source of genome instability and cancer. In this regard, the frequency of telomere-to-telomere fusion events per cell found in this work is extremely high, if we consider multicellular organisms. It is tempting to speculate that spontaneous chromosome fusions also occur in higher eukaryotes, even if most should result in senescence or cell death (Shay and Wright, 2011; Suram et al., 2012).

Spontaneous telomere-to-telomere fusions may also give a plausible explanation for specific evolutionary events. Human chromosome 2 is thought to be the result of a telomere fusion between two independent chromosomes, which were retained in the chimpanzee (Fan et al., 2002; Ijdo et al., 1991; Lejeune et al., 1973; Park et al., 2000; Yunis and Prakash, 1982). If telomere protection becomes overall compromised in the germline, there is little chance of generating viable offspring. However, a single spontaneous telomere-to-telomere fusion could occur in an otherwise healthy germline cell, which could be viable and become fixed in a population. In the case of human chromosome 2, the fusion junction

between the ancestral chromosomes is still visible in the form of telomere sequences in a head-to-head orientation (Ijdo et al., 1991). Since there is some degeneration in these sequences, it is impossible to determine whether the intervening telomeres were critically short. However, it is possible that this event resulted from a stochastic telomere-to-telomere fusion in otherwise functional telomeres. Similar "telomeric artifacts" of this kind have been found in several tetrapods, including the armenian and chinese hamsters, several moles, the wallaby *Macropus rufogriseus*, and the lizard *Gonatodes taniae* (Ashley and Ward, 1993; Meyne et al., 1990; Moyzis et al., 1988; Schmid et al., 1994). In the house mouse, many Robertsonian fusions are known to have created metacentric chromosomes from the fusion of two separate telocentric chromosomes (Garagna et al., 1995; Garagna et al., 2001; Nanda et al., 1995). However, in these cases, telomeric sequences have been lost leaving only near microhomologies of satellite DNA at the fusion junctions. This suggests that these fusions may have been the result of critical telomere shortening or of DNA repair that lead to loss of telomere sequences during fusion. Curiously, these robertsonian fusions were responsible for the rapid reproductive isolation of the house mouse in Madeira, generating six distinct races of mouse in less than 500 years (Britton-Davidian et al., 2000).

It would be therefore important to test the occurrence of spontaneous telomere-to-telomere fusions in multicellular organisms. For that end, a similar positive detection assay could be designed for natural chromosome ends, using, for example, the expression of a fluorescent marker as an indicator of telomere fusions.

3.2 - The telomere capturing assay delivers an underestimated value for telomere fusions

It is likely that the described system is unable to detect all ongoing fusions in WT and *trt1*Δ backgrounds. Detection of fusions relies on the reconstitution of the *his3*$^{+}$ gene. However, there are several possible end-to-end fusion reactions which would be unproductive, i.e., fail to reconstitute *his3*$^{+}$ expression. These could involve (i) the destruction of the coding sequence or intronic splice sites of the reporter gene; (ii) fusions between mini-chromosome and endogenous chromosome ends; (iii) fusions between mini- chromosomes that do not pair opposite ends or even (iv) the generation of linear *trt1*Δ survivors that continuously undergo HR repair at the chromosome ends. In this regard, liquid cultures of *trt1*Δ strains are dominated by linear survivors (Nakamura et al., 1998; Subramanian et al., 2008). This could explain why only 5%-8% of *trt1*Δ cultures are constituted by revertant cells. In fact, Southern blot analysis of a *taz1*$^{+}$ *o/e trt1*Δ strain carrying pFT2 shows the reemergence of weak telomere signals after critical telomere erosion (Figure 2.21). Thus, for all backgrounds the actual number of fusions is likely underestimated.

Subsequent detection of unproductive fusions is a challenge, since they are not detected by *his3*$^{+}$ expression. Additionally, our work shows no evidence of productive intermolecular fusions, i.e., fusions between independent copies of pFT2 which result in reconstitution of *his3*$^{+}$. Nevertheless, multimers of pFT2 within revertant clones could be screened by Southern blot. Further experiments could assess whether there is a bias towards intramolecular or intermolecular fusions. Such a bias would suggest that spatial proximity is an important factor in telomere-to-telomere fusions. If so, then the frequency of telomere fusions should vary with copy number of pFT2. This effect could be discarded by introducing the telomere fusion capturing system at an endogenous chromosome. Alternatively, the

promoter-proximal and the terminator-proximal regions of the *his3*$^{+}$ gene could be separated between different plasmids.

The frequency of detected fusions could be further influenced by pFT2's stability in the linear and circular configurations. In fission yeast, adding internal or terminal telomeres to a plasmid increases its loss rate (Figure 2.8). WT cells carrying pFT2 suffer from cold sensitivity, which is rescued when Taz1 is overexpressed (Figure 2.19). Thus, it is possible that replication fork stalling events increase loss of pFT2 in a cell culture. As such, further experiments could reveal whether overexpressing Taz1 rescues this phenotype.

Internal telomere tracts improve the segregation of circular plasmids in budding yeast (Longtine et al., 1992). However, this study was performed with direct telomere repeats. Inverted telomere repeats, like the ones in circular versions of pFT2, are reported to generate improper DNA structures in higher eukaryotes, which create fork stalls during replication and genomic instability (Alvarez et al., 1993; Bosco and Lange, 2012; Fernandéz et al., 1995; Kilburn et al., 2001; Slijepcevic et al., 1996). Thus, if the circular configuration of pFT2 has improved segregation, this may be counterbalanced by its inherent instability.

The majority of linear pFT2 plasmids were not immediately stabilized once inside cells. From the results presented here, we conclude that naked telomere sequences are not immediately recognized as telomeres, once inside the cell. Transforming linear plasmids into fission yeast cells resulted in at least 78% frequency of plasmid-end fusions, judged by the expression of *his3*$^{+}$ clones retrieved after transformation. Only 4% of the analyzed transformants were able to effectively recruit Shelterin to pFT2's telomeres. Telomere ends are recognized and processed as DNA breaks usually when shelterin is incomplete or absent (Sfeir and de Lange, 2012). As discussed in section 1.4.6, telomere

protection depends on the DDR machinery as much as it depends on Shelterin. A complex relationship between the two establishes how telomeres are processed during every cell cycle, without compromising their integrity. Accordingly, it is known that the recruitment of Shelterin components is cell cycle-dependent (Flynn et al., 2011; Gilson and Géli, 2007; Moser et al., 2009a; Verdun et al., 2005). It is most probable that recognition of telomere ends requires an orderly sequence of events, from DNA-end processing to recruitment of specific Shelterin components, which may not be recruited at any phase of the cell cycle. This would explain the overwhelming majority of "failed" transformations of the linearized version of pFT2.

3.3 - Telomere length homeostasis: a matter of dosage

Telomere length is a species-specific trait that varies widely between organisms. They range from short 300 bp in yeast to as long as 15-20 Kbp in humans and between 25 and 150 Kbp in laboratory mice (Gomes et al., 2011; Kipling and Cooke, 1990; Matsumoto et al., 1987; Sherr and DePinho, 2000). Despite this variability, it is a tightly regulated feature within each species (Gomes et al., 2011; Marcand et al., 1997; Miller et al., 2005; Teixeira et al., 2004). However, telomere length is the result of a dynamic equilibrium, as individual telomere length changes at every cell cycle. These changes mostly reflect the incomplete replication of chromosome ends, and the compensating effect of telomere repeat addition by telomerase. Other contributing factors include telomere resection by nucleases, telomere rapid deletion (TRD) effects, and homologous recombination (Bucholc et al., 2001; Li and Lustig, 1996; Stewart et al., 2012). Telomerase activity or processivity may be at the source of major differences in telomere size. For example, while WT

telomeres are elongated specifically during late S phase, *taz1*Δ telomeres are elongated by telomerase throughout the cell cycle, resulting in a dramatic increase in telomere length (Dehé et al., 2012). In this regard, it is curious to note that while excluding hTRF1 from human telomeres also results in telomere elongation (Van Steensel and de Lange, 1997), deleting mTRF1 from laboratory mice, which are known for having considerably longer telomeres than wild-derived mouse strains (Hemann and Greider, 2000), has no detectable effect in telomere length (Iwano et al., 2004). Perhaps differences in telomere length between mouse strains could be attributable to differences in telomerase regulation by mTRF1. Testing whether a mTRF1 copy from a wild-derived mouse strain reduces telomere length in a laboratory mouse strain may reveal whether their differences in telomere length are attributable to a genetic change in mTRF1.

Similar to what was reported in budding yeast (Runge and Zakian, 1989), our work shows that doubling the number of telomeres in fission yeast cells led to a substantial increase in telomere size. This phenotype was compensated by overexpressing Taz1, and partially compensated by overexpressing Pot1 (Figure 2.18), which may become limiting after a significant increase in telomere size. This observation is in accordance with a previous report in fission yeast showing that reducing telomere Pot1 binding through competition with a dominant mutant results in telomere elongation (Bunch et al., 2005). In addition, human hPOT1 was also implicated in hTRF1-mediated regulation of telomerase (Loayza and de Lange, 2003). It would therefore be of interest to test whether Pot1 overexpression reduces any other *taz1*Δ-related phenotypes.

Limiting amounts of Taz1 cause the average telomere length to increase from ~300 bp to ~1 Kb (Figure 2.14), which is still considerably shorter than *taz1*Δ telomeres (~3 Kb; (Ferreira and Cooper, 2001). The most striking phenotype of *taz1*Δ cells is perhaps the occurrence of NHEJ-

mediated telomere-to-telomere fusions in G_1-arrested cells. For WT strains carrying pFT2, which are insufficient for Taz1, I was unable to detect an increase in histidine-producing cells after a G_1-arrest. However, in cycling cultures, this strain possesses a higher frequency of spontaneous telomere fusions than the *taz1*$^+$ *o/e* strain (Figure 2.28), confirming that they are more susceptible to telomere fusion reactions. In addition, Taz1 deficiency resulted in a cold sensitive phenotype, as it was observed for *taz1*Δ mutants (Figure 2.19; (Miller and Cooper, 2003). This phenotype has been associated with replication fork stalling at telomeres, which in *taz1*Δ mutants leads to defective telomere replication and fork collapse (Miller et al., 2006). Consequently, it is thought that *taz1*Δ telomeres are lost and resynthesized at every cell cycle (Dehé et al., 2012). However, in our work sequencing of telomere-to-telomere fusions shows that the original telomere sequences introduced in pFT2 were not lost, and in some cases were retained for over 200 bp (Figure 2.36). Thus, even though they were subjected to conditions of Taz1 deficiency, they did not possess all the characteristics of a full *taz1*Δ mutant.

Telomere length has been artificially manipulated in other organisms. Overexpression of telomerase and its RNA subunit in human cells resulted in a 3-8 fold increase in telomere length (Cristofari and Lingner, 2006). Increasing quantities of telomerase may overwhelm the inhibitory role of hTRF1. Accordingly, overexpression of hTRF1 or hTRF2 in telomerase-positive cells results in gradual telomere shortening (Karlseder, 2002; Van Steensel and de Lange, 1997). Two-fold concentrations of Taz1 in the *taz1*$^+$ *o/e* strain did not lead to telomere shortening below WT levels (Figure 2.14). However, plasmid-based overexpression of Taz1 resulted in telomere shortening marginally below the control (Figure 2.18). It remains to be tested whether Taz1 expression could yield more dramatic results if the gene were to be placed under a stronger promoter.

Finally, it seems that telomere length is a malleable trait, which may be subjected to alterations without significantly compromising viability. In this regard, the variability in telomere lengths found in nature could not only result from differences in telomerase regulation, but also from changes in the number of chromosomes. These would unbalance the ratio between telomeres and telomere length regulators and modify steady-state telomere length without altering the basic mechanism of regulation.

However, not all changes in expression of telomere components are benign. In the system described here, Rap1 overexpression lead to telomere instability (Figure 2.18). A similar effect is observed when overexpressing ScRap1 in budding yeast (Conrad et al., 1990). In this case, instability was accompanied by abnormal telomere elongation, which were undetected in our experimental conditions of Taz1 deficiency. Because it mediates the connection between dsDNA- and ssDNA telomere binding proteins (Miyoshi et al., 2008), excess Rap1 could deprotect telomeres by tethering shelterin components away from the telomeres. Alternatively, increasing Rap1 concentration could promote abnormal telomere recombination and instability, since Rap1 is required for recombination-mediated telomere maintenance in *trt1*Δ and *trt1*Δ *taz1*Δ cells (Subramanian et al., 2008). This function is independent of its recruitment to telomeres by Taz1. In fact, recombination is inhibited by Taz1 and Trt1. *In vitro* experiments using budding yeast's ScRap1 further support its role in promoting recombination between telomere sequences (Gilson et al., 1994). Thus, we speculate that Rap1's recombinatorial function may no longer be fully inhibited when overexpressed.

Overexpression of Ccq1 also resulted in telomere rearrangements (Figure 2.18). This was surprising, since Ccq1 is a checkpoint inhibitor and its overexpression abolishes the telomere entanglement defect seen in *taz1*Δ cells (Motwani et al., 2010; Tomita and Cooper, 2008). Interestingly,

Ccq1 also recruits the transcriptional gene silencing complex SHREC to telomeres (Sugiyama et al., 2007). It is, therefore, possible that abnormal quantities of Ccq1 could alter heterochromatin profiles, by sequestering SHREC away from telomeres and disrupting transcriptional silencing. Thus, imbalances in the cellular content of Shelterin components may be detrimental to telomere protection.

An additional factor that may influence telomere length is transcription. Southern blot analysis of pFT2 suggests that transcription at telomeres has a positive effect in telomere length. While the promoter-proximal telomere (T Prom) should be transcribed via the *his3*$^{+}$ promoter, the terminator-proximal telomere (T Term) is not expected to be transcribed. Coincidentally, T Term has shorter telomeres on average than T Prom or the endogenous telomeres, which are also subjected to transcription (Greenwood and Cooper, 2012). Northern blot analysis to investigate whether *his3*$^{+}$-telomere tract transcripts are produced was not performed. Nevertheless, it is possible that the transcription machinery, engaged by the *his3*$^{+}$ promoter, opens telomeres to telomerase, resulting in longer telomeres. However, a previous report shows that cloning an exogenous promoter in the direction of budding yeast telomeres results in telomere shortening, rather than elongation (Sandell et al., 1994). Recently, it was shown that increasing TERRA levels in budding yeast induces telomere shortening through telomere resection by Exo1 (Pfeiffer and Lingner, 2012). These results are contrary to what is documented in human cells, in which telomere length is unaffected by increasing transcription of TERRA (Farnung et al., 2012). Collectively, these results suggest that telomere transcription has variable functions and effects in different model organisms (Bah and Azzalin, 2012). Alternatively, transcription through the telomere tract may result in telomere lengthening or shortening, depending on the strength of the promoter driving transcription.

3.4 - DNA hairpins may momentarily stabilize eroding chromosome ends

In contrast to the telomere-to-telomere fusions of unperturbed cells, fusions in the absence of telomerase were SSA-mediated. Choice of DSB repair may reflect telomere length and the availability of microhomologies. While Shelterin protects telomeres from 5'-strand resection required for SSA-mediated repair (Pitt and Cooper, 2010), telomere erosion in telomerase mutants exposes ssDNA, which inhibits NHEJ and may serve as substrate for SSA repair (Dimitrova and de Lange, 2009).

SSA-mediated repair had already been implicated in *pot1*Δ and *trt1*Δ chromosome-end fusions (Wang and Baumann, 2008). However, in these cases, fusions occur after massive degradation of chromosome ends up to 13 Kb, using imperfect homologies that extend between ~300-800 bp (Nakamura et al., 1998; Wang and Baumann, 2008). In contrast, fusions captured by the pFT2 plasmid in *trt1*Δ mutants occur through the use of pentanucleotide microhomologies that are contiguous to telomeres (Figure 2.23). These pentanucleotides are arranged as inverted repeats that could fold back when resected, generating DNA hairpins. The MRN complex and its partner Ctp1 were required for fusions in the absence of telomerase (Figure 2.27). Thus, hairpin structures may stabilize eroding 3'-strands, which must be subsequently processed by MRN and Ctp1 in order to fusions to occur (Helmink et al., 2011; Lengsfeld et al., 2007; Lobachev et al., 2002). MRN was previously implicated in fusions involving critically short telomeres in Arabidopsis and human cells (Heacock et al., 2004; Tankimanova et al., 2012). In both cases, defects in Mre11 result in decreased microhomology-mediated fusions, and in the case of Arabidopsis *mre11* mutants, no fusions were observed within subtelomeric regions. A similar requirement for MRN was found in telomerase RNA mutants in budding yeast (Chan and Blackburn, 2003). However, MRN is

dispensable for SSA-mediated chromosome-end fusions in *pot1*Δ fission yeast mutants (Wang and Baumann, 2008). I was unable to find inverted repeats that could form hairpins within the fusion sequences provided by Wang and Baumann. Thus, it is possible that MRN is specifically required for hairpin processing in the pFT2-captured fusions.

Therefore, the fusions here described may represent a subset of fusion reactions occurring in *trt1*Δ mutants, which had remained undetected until now. However, it is possible that these types of fusion do not occur in *pot1*Δ mutants. Removal of Pot1 results in immediate 5'-strand telomere degradation (Miller et al., 2006). This may lead to replication fork collapse due to the repetitive nature of telomeric ssDNA, thus removing the pentanucleotide sequences present at the 3'-strand (Pitt and Cooper, 2010; Rog et al., 2009).

The preference for these pentanucleotides to engage in fusion reactions remains to be explained. The role of hairpins in telomere fusions should be further assessed by destroying inter-strand complementarity, while keeping homology between these sequences. If hairpins dictate the sequences involved in repair, we should either abolish the appearance of histidine-producing colonies, or observe the emergence of new patterns of end fusion.

3.5 - Future applications of the telomere fusion-capturing assay

The telomere fusion-capturing assay has potential applications beyond the characterization of telomere dysfunction outcomes. The most promising prospect is the use of pFT2 in screening for telomere dysfunction mutants. Mutants with unprotected telomeres are expected to yield a higher frequency of histidine-producing cells than background spontaneous fusions. For screening purposes, a library of mutants could be generated

through a random mutagenesis approach. The transposon-based mutagenesis described by Park and co-workers has two main advantages (Evertts et al., 2007; Park et al., 2009): i) it can disrupt coding sequences by randomly inserting a modified *Drosophila* transposon into the genome and; ii) the disrupted regions can be identified by sequencing, using specific transposon sequences as primer DNA. Nevertheless, this method may only be able to identify *est*-like mutants, since disruption of *pot1*$^{+}$ in strains carrying pFT2 did not result in increased expression of histidine.

3.6 - Concluding remarks

Telomeres have been discovered to be complex chromosomal domains, with several related, but separable functions. In addition, it has been suggested that Shelterin imposes changes in telomere architecture, thereby controlling many of its functions, including telomere length regulation and chromosome-end protection (de Lange, 2005; Marcand et al., 1997). Given this, there has been increasing focus on the dynamics of telomere structure. In order to protect chromosome ends from degradation and fusion, telomeres need to be recognized as DNA damage in every round of replication, which seems paradoxical and akin to "playing with fire". Generally, it is thought that replicating telomeres are reinstated as fully insulated structures without incident. The results here presented suggest that this is not always the case. By their very nature, telomeres may occasionally be subjected to undue repair, which they are unable to thwart. Telomere protection is not infallible.

In addition, if occurring in otherwise healthy cells, spontaneous telomere-to-telomere fusions could result in genomic instability and cancer. Therefore, investigating the occurrence of these events in higher eukaryotes should be an exciting subject for future research. For example,

if cancer is an "old age disease" (Pereira and Ferreira, 2012), then are there benign telomere-to-telomere fusions occurring throughout early life? Do these fusions increase with age?

It remains to be determined whether these telomere-to-telomere fusions are unstable and re-open, as it was previously observed for budding yeast (Lundblad and Szostak, 1989; Pobiega and Marcand, 2010). This could constitute a pathway for the rescue of telomere fusions, which restores normal karyotype.

Furthermore, these results show that telomeres are subjected to different kinds of repair, depending on length. Fusions after telomere erosion are mediated by microhomology-dependent mechanisms of end-joining. Instead, telomere-to-telomere fusions use NHEJ repair. Thus, both mechanisms seem to be biologically relevant for telomere dysfunction, although in different conditions.

The MRN pathway was found to play a role in both of these fusion pathways. However, previous reports suggest that its function is not the same for both cases. In fission yeast, MRN is not required for NHEJ repair of DSBs (Manolis et al., 2001). However, NHEJ-mediated telomere-to-telomere fusions in *taz1*Δ cells require MRN, supposedly for tethering DNA ends together (Reis et al., 2012; Williams et al., 2008). Thus, MRN should be required for spontaneous telomere fusions also for bridging DNA ends towards fusion. On the other hand, *pot1*Δ cells undergo SSA-mediated chromosome-end fusions which do not require MRN (Wang and Baumann, 2008). In the case of fusions captured by pFT2 upon telomere erosion, MRN may be required for processing of hairpin structures formed upon resection of the 5'-strand, in a Ctp1-dependent manner.

In conclusion, this work revealed a potentially new source of genomic instability, while clarifying the nature of DNA repair involved in telomere fusions in different genetic conditions. Future studies will be able

to assess the relevance of these observations through additional experiments, and in other model organisms.

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Appendices

Appendix A. Three specific pentanucleotides, present at subtelomeric sequences, are used in *rad16*$^{+}$-dependent fusions Sequence of original subtelomeric and telomeric DNA cloned inside the *his3*$^{+}$ intron. Subtelomeric sequences are in lower case, and telomeric sequences are in upper case. Pentanucleotide sequences used in *rad16*$^{+}$-dependent fusions are underlined.

T PROM:

5′-tctagacgtcagccgagccgtaaggcgaggctgcgggttacatacaggttacgggccccacccgtcagccgagccgtaaggcgag
gctgcgGGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGGTTACAGGGGTTACGGT
TACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTTACAGGGGTTACGGTTACACGGTTACAG
GGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGGTACGGTTACAGGGGGGGGTTACGGTTACAGGGGTTACAGGG
GG-3′

T TERM:

3′-CCCCCCTGTAACCCCTGTAACCGTAACCCCCCCCCTGTAACCGTACCCCTGTAACCCCCTGTAACCTGTAACCCCTGTAACCCTGT
AACCCCCTGTAACCGTGTAACCGTAACCCCTGTAACCCTGTAACCGTGTAACCCTGTAACCGTAAACTGTAACCGTGTAACCTGTAAC
CCTGTAACCGTAACCCCTGTAACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC
cgcagcctcgccttacggctcggctgacgggtggggccctgtaacccctgtaaccgtaacccccccctgtaaccgtaccctgtaact
ctaga-5′

Appendix B. Examples of microhomology-mediated fusions in pFT2, captured in *trt1Δ* and *trt1Δlig4Δ* survivors.

C/I type fusion:

(T PROM)tctagacgtcagccgagccgtaaggcgaggctg**cgggt**ggggccctgtaacccctgtaaccgtaacccccccctgta
accgtacccctgtaactctaga(T TERM)

B/H type fusion:

(T PROM)tctagacgtcagccgagccgtaaggcga**ggctg**acgggtggggccctgtaacccctgtaaccgtaacccccccctgt
aaccgtacccctgtaactctaga(T TERM)

A/G type fusion:

(T PROM)tctagacgt**cagcc**ctcgccttacggctcggctgacgggtgggg(T TERM)

F/I type fusion:

(T PROM)tctagacgtcagccgagccgtaaggcgaggctgcgggttacatacaggttacgggccccacccgtcagccgagccgt
aaggcgaggctg**cgggt**ggggccctgtaacccctgtaaccgtaacccccccctgtaaccgtacccctgtaactctag
a(T TERM)

E/H type fusion:

(T PROM)tctagacgtcagccgagccgtaaggcgaggctgcgggttacatacaggttacgggccccacccgtcagccgagccgt
aaggcga**ggctg**acgggtggggccctgtaacccctgtaaccgtaacccccccctgtaaccgtacccctgtaactcta
ga(T TERM)

D/G type fusion:

(T PROM)tctagacgtcagccgagccgtaaggcgaggctgcgggttacatacaggttacgggccccacccgt**cagcc**tcgcctt
acggctcggctgacgggtggggccctgtaacccctgtaaccgtaacccccccctgtaaccgtacccctgtaactcta
ga(T TERM)

Appendix C. NHEJ-mediated fusions captured in *taz1*$^{+}$ *o/e* + pFT2 cells. Shown are telomeric sequences found at telomere-to-telomere fusions in *taz1*$^{+}$ *o/e* cells. Originally cloned sequences are in black, and sequences added *in vivo* are in red. "/" marks the point of fusion between the promoter-proximal (T Prom) and the terminator-proximal (T Term) telomeres.

Fusion 1 (found in 2 clones):
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGG
GTTACAGGGGGTTACGG /
CCACGTAACCTTGTAACC-3′:T TERM

Fusion 2:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGG
GTTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGG
TTACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGG
TACGGTTACAGGGGTTACAGGGGTTACGGTTACAGTTACAGGTTACGGTTACAGGTTACAG /
CTGT
AACCTGTAACCGTGTAACCCTGTAACCGTAAACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTAA
CCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 3:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGTTACAGGGG
TTACAGGTTACAGGGGTTACGGTTACAGGGGTACGGTTACAGGTTACAGGGTTACAGGTTACAGGGTTACAGGGGGT
TACGGTTACGGTTACCGGTTACCGTTACAGGGGTTATAGGGGTTACC /
GT
AACCCCTGTAACCGTAACCCCTGTAACCGTGTAACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 4:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGGGTTACACGGTTACAGGGTTACAGGTTACAGGG
GGTACGGTTACAGGTTACAGGTTACAGGTTACAGGTTACAGGGGGTTACAGGTTACAGGTTACGGTTACAGGGTTACG
GTTACAGGTTACAGGGGGTTACAGGGGTTACGGTTACAGGTTACAGGTTACAGGGGGGTTACAGGGTTACAGGGGTT
ACGGTTACACGGTTACAGGGTTACGGTTACAGGGGGGTTACAGGGTTACGGTTACAGTTACAGGGTTACACGGTTAC
AGGGGTTACGGTTACAG /
GTGTAACCCTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 5:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACCCGGTTACAGGGTT
ACACGGTTACAGTTACAGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGTTACG /
CTGTAACCCTGTAACCTGTAACCGTAACCCCCTGTAACCTGTAACCGTACCCCTGTAACCGTAACCGTA
ACCCTGTAACCCTGTAACCTGTAACCTGTAACCTGTAACCCCTGTAACCCCTGTAACCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 6:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGGTAC
GGTTACAGGGGTTACCGG /
CTGTAACCGTAAACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGT
AACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAAC-3′:T TERM

Fusion 7 (found in 2 clones):
T PROM: 5′-GGTTACAAGGTTACGTGGTTACCCGGTTACAGGTTACAGGTTACAG /
CGTAACCTTGTAACC-3′:T TERM

Fusion 8 (unknown sequences at fusion junction):
T PROM: 5′-
GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGTTACAGGGTTACAGGTTACAGTTACAG
GGTTAC /
ATGTCAGCAATACTACACTACGCTATAATACACTACGTTGAGTATCACTATATGTCACATGTTCTAATTATATATCG
TACCATGTATGATACGATATGGAGATTGATCTTAATGATAATCTATTAAGATCAATATTATCTGAATACTATAAATA
GAGCTACTGCTGAACCTCGTTCCTCAGTTC /
GTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 9:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGGTTACGGTTACGGTTACAGGGGTACGGTTACAGGGGTTACAGGGTTACAGGGGTTACAGGGTTACAGG
GTTACAGGTTACGGTTACAGGTTACACGGTTACAGGGGTTACGGTTACAGGTTACAGGGGGTTACAGGGGTTACAGG
TTACA /
TGT
AACCTGTAACCCCTGTAACCGTAACCCTGTAACCCTGTAACCTGTAACCTGTAACCGGTGTAACTGTAACCTGTAAC
CCCCCCTGTAACCCTGTAACCTGTAACCTAACCCCTGTAACCCCTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 10:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACAGGTTACAGGTTACGGTTACAGGTTACAGGTTACAGGTTACAGGGGGGTTACAGGTTACAGGTT
ACACGGTTACACGGTTACAGGGTTACGGTTACAGGGGTTACAGGTTGGTTACACGGTTACAGGGTTACGGTTACAGG
TTACGGTTACAGTTACGGTTACAGTTACAGGTTACAGGTT /
TGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 11:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACACGGTTACAGGGGTTACGGTTACAGGGTTACAGGGGGGTTACAGGTTACAG
GTTAGGTTACAGGTTACGGTTACAGGGGTTACGGTTACAGGTTACGGTTACGGTTACAGGGGTTACAGGTTACGGTT
ACAGGGGTTACAGGTTACAGGGGTGTTACAGGGGGGTTACAGGTTACAGGTTACAGGGGGGTTACAGGTTACAGGGG
TTACGGTTACAGGGGGTAACAGGTTACAGGGGGGGTTACGGGG /
CTGTAACCCTGTAACCCCCTGTAACCGTGTAACCGTAACCCCTG
TAACCCTGTAACCGTGTAACCCTGTAACCGTAAACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 12:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGGGTTACAGGTTACAGTTACAGGGGTTACAGGTTACAGGGTACGGT
TACA /
NO TELOMERES-3′:T TERM

Fusion 13:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGGGTTACGGTTACGGTTACAGGGGTTACGGTTACAGGTTACAGGGGGTT
ACAGGGGGTTACAGGGGGGTTACAGGTTACACGGTTACAGGGGTTACAGTTACAGGTTACCGGTTACAGGGTACGGT
TACAGTTACGGTTATGGGGTTACAGGTTACAGGGTTACA /
AGTAACCTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 14:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACAGGGTTACAGGGTTACAGTTACAGGGTTACGGTTACAGGTTAC
AGGTTACAGGGTTACGGTTACAGGGTACGGTTACAGGGGGGGGGGTTACAGGGGTAACGGTTACAGGTTACAGGTTA
C /
CTGTAACCCCCTGTAACCGTACCCTGTAACTGTAACCCCTGTAACCGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 15:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACAGGTTACAGGGGGGTACGGTTACAGGTTACAGGGGGGTTACGGT
TACGGTTACGGTTACACGGTTACAGGGGTACGGTTACAGGGGTTACAGGGTTACGGTTACGGTTACAGGGTTACAGG
GTTACAGGTTACAGTTACAGGGTTACACGGTTACAGGTTACAGG /
GTAA
CCCCCTGTAACCTGTAACCCTGTAACCCCTGTAACCGTACCCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 16:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGGGTTACAGG /
CTGTAACCTGTAACCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 17 (found in 2 clones):
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTACAGGGGTTACGGTTACGGTTACACGGTTAC
AGGGGGGTTACAGGGTTACGGTTACAGTTACGGTTACAGGGGGTTACGGTTACAGGTTACAGGTTACGGTTACAGGG
TTACAGGTTACAGGGTTACAGGGGGTTACGGTTACAGGGTTACGGTTACAGGGTTACAGGTTACAGGTTACAG /
TGTAACCCTGTAACCCTGTAACCTTGTAACCT
GTAACCCTGTAACCCCCTGTAACCTGTAACCCCTGTAACCGTACCCCCTGAACCGTAACCGTGTAACCCCCTGTAAC
CTGTAACCTGTAACCGTAACCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 18:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACACGGTTACAGGGTTACAGGTTACAGGGTTACGGTTACAGGGGTTACAGGGGGTTACAGGGGTTACAGGTTACAGG
GTTACAGTTACACGGTTACACAGGTTACAG /
GTAACCCCCCCCCCCCTGTAACCGTGTAACCTGTAACCCCCTGTAACCGTAA
CCCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 19:
T PROM: 5′-
GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGGTTACAGGGGTT
ACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTTACACGGTTACAGGT
GTTACAGGGGTTACAGTTACAGGTTACAGGGTTACAGGTTACAGGGGTTACGGTTACAGGGTTACACGGTTACAGGT
TACAGGGGGTTACACGGTTACAGGTTACGGTTACACGGTTACAGGGTTACGGTTACAGGGGTTACAGA /
ACCTGTAACCGTAACCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 20:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGGTTACAGGTTACGGTTACAGGTTACGGTTACAGGGGGTACGGTTACAGGTTAC /
TG
TAACCGTGTAACCTGTAACTGTAACTGTAACCTGTAACCTGTAACCTGTAACCGTAACCTTGTAACC-3′:T TERM

Fusion 21:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACCGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACTGGGTTACAGGGGTTACAGGTTACAGGGGGGTTACAGGTTAC
/
AACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCCTGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 22:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGGTAC
GGTTACAGGTTACAGGTTACAGGGTTACAGGGTTACGGTTACAGGTTACAGGGGGGTTACAGGTTACAGGGGGTTAC
AGGGGTTACACGGTTACAGGGGTACG /
GTAACCCGTAACCGTGTA
ACCGAACTGTAACCGTAACCGTACCCCTGTAACCCTGTAACCCCTGTAACCGTAACCGTAACCCCCCTGTAACCGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 23:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGGTAC
GGTTACAGGTTACAGGTTACAGGGTTACAGGGTTACACGGTTACAGGTTACAGGTTACAGGTTACA /
GTGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

Fusion 24:
T PROM: 5′-GGTTACAAGGTTACGTGGTTACACGGTTACAGGTTACAGGTTACAGGGGGGTTACGGTTACAGGGG
TTACAGGGGTTACGGTTACAGGGTTACAGGTTACACGGTTACAGTTTACGGTTACAGGGTTACACGGTTACAGGGTT
ACAGGGGTTACGGTTACACGGTTACAGGGGGTTACAGGGTTACAGGGGTTACAGGTTACAGGGGGTTACAGGGGTAC
GGTTACAGGGGTTACCGG /
CTGTAACCGTAAACTGTAACCGTGTAACCTGTAACCCTGTAACCGTAACCCCTGTA
ACCCCTGTAACCGTAACCCCCCCTGTAACCTGTAACCTGTAACCGTGTAACCACGTAACCTTGTAACC-3′:T TERM

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Spontaneous telomere to telomere fusions occur in unperturbed fission yeast cells

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ABSTRACT

Telomeres protect eukaryotic chromosomes from illegitimate end-to-end fusions. When this function fails, dicentric chromosomes are formed, triggering breakage-fusion-bridge cycles and genome instability. How efficient is this protection mechanism in normal cells is not fully understood. We created a positive selection assay aimed at capturing chromosome-end fusions in *Schizosaccharomyces pombe*. We placed telomere sequences with a head to head arrangement in an intron of a selectable marker contained on a plasmid. By linearizing the plasmid between the telomere sequences, we generated a stable mini-chromosome that fails to express the reporter gene. Whenever the ends of the mini-chromosome join, the marker gene is reconstituted and fusions are captured by direct selection. Using telomerase mutants, we recovered several fusion events that lacked telomere sequences. The end-joining reaction involved specific homologous subtelomeric sequences capable of forming hairpins, suggestive of ssDNA stabilization prior to fusing. These events occurred via microhomology-mediated end-joining (MMEJ)/single-strand annealing (SSA) repair and also required MRN/Ctp1. Strikingly, we were able to capture spontaneous telomere-to-telomere fusions in unperturbed cells. Similar to disruption of the telomere regulator Taz1/TRF2, end-joining reactions occurred via non-homologous end-joining (NHEJ) repair. Thus, telomeres undergo fusions prior to becoming critically short, possibly through transient deprotection. These dysfunction events induce chromosome instability and may underlie early tumourigenesis.

INTRODUCTION

The ends of eukaryotic chromosomes prevent DNA double-strand break (DSB) repair through a specialized protective structure called the telomere. Telomeres are composed of G-rich DNA repeats bound by a protein complex known as shelterin (1,2). In the absence of shelterin, telomeres undergo end-to-end fusions that give rise to genomic instability (3–5). Several studies suggest that genomic instability initiated by telomere dysfunction may underlie carcinogenesis (6).

The functions of telomere protection have been dissected in several organisms from yeast to humans. One major function is to compensate for the incomplete replication of the ends of chromosomes, known as the 'end replication problem' (7). This is generally achieved by telomerase, a reverse transcriptase that adds new telomere repeats to the ends of chromosomes (8–10). In the fission yeast *Schizosaccharomyces pombe*, telomere elongation by telomerase is regulated by Taz1, orthologue of both human TRF1 and TRF2, also involved in chromosome-end protection (2,11,12). In absence of Taz1, telomere-to-telomere fusions occur via non-homologous end-joining (NHEJ) repair, a mode of DSB repair up-regulated in the G1 phase of the cell cycle (2,11,12). Similarly, disruption of either human TRF2 or budding yeast Rap1 results in telomere-to-telomere fusions perpetrated by NHEJ repair (13,14). During DNA replication, the replication fork unwinds the telomere and exposes chromosome ends. While this permits the engagement of telomerase, it also causes momentarily deprotection that triggers DNA damage checkpoints (15,16).

Although loss of the telomere repeat factor Taz1 triggers NHEJ-mediated fusions, erosion of telomeric DNA leads to a different mode of DNA repair. In the absence of telomerase, Stn1, Ten1 or Pot1—components that protect the 3′-overhangs from degradation (17)—chromosome ends undergo fusions through a DNA repair process that requires exposure of complementary ssDNA regions on both DNA ends (18). Depending on the size of the homology region involved, this process has been termed single-strand annealing (SSA) or microhomology-mediated end-joining (MMEJ) repair (19). In fission yeast, these fusions normally occur 7–13 kb internally after complete disappearance of telomere sequences (20). In mouse cells, a microhomology-dependent mechanism of DNA repair termed A-NHEJ also mediates fusions after

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removal of Pot1a/b-Tpp1 (21). Similar results were obtained for fusions observed in telomerase-deficient budding yeast and mice models (3,22). Chromosome-end fusions were also observed in telomerase-positive human cell lines (23). Most involve critically short telomeres, suggesting that telomeres fuse after eroding past a minimum length required for protection. In contrast to these studies, wild-type length telomeres can engage end fusions. Gottschling and colleagues used a positive selection assay in budding yeast based on an induced DSB and observed extremely rare telomere-to-DSB fusions in WT cells, highlighting that normal telomeres are protected from engaging in NHEJ repair with other DNA breaks (24).

Here we investigate the nature of telomere protection in fission yeast using a novel quantitative telomere fusion assay. We find that telomerase mutants show microhomology-dependent fusions possibly mediated by transient DNA hairpin structures. In contrast to previous observations, we show that wild-type telomeres are subjected to spontaneous fusions via NHEJ, which do not require critical telomere shortening. Thus, functional telomeres can be involved in spontaneous telomere fusions, which may trigger genomic instability preceding carcinogenesis.

MATERIALS AND METHODS

Mini-chromosome and plasmid construction

pFT2

The *his3*$^+$ gene was cloned into pBluescriptII KS(+) using a blunt-ended NotI/SmaI digestion. A synthetic 100 mer oligonucleotide containing subtelomeric sequences in a head-to-head orientation, derived from the pNSU70 plasmid (25) separated by an ApaI restriction fragment was cloned into the XbaI restriction site of *his3*$^+$. The resulting *his3*$^+$ fragment was then removed from pBluescriptII using PstI and SacI and cloned into the PstI and SacI sites of pREP3X. The SacI site was subsequently destroyed by digestion and blunt-end ligation to generate pREP-his-telo. Subsequently, an ApaI restriction fragment containing opposing telomere sequences separated by an *Escherichia coli* kanR resistance gene was derived from the pEN53 plasmid (26) and cloned into the ApaI site, thus spacing the subtelomeric sequences of pREP3X-his-telo to give pFT2. Linearization of the plasmid was achieved by removing the SacI-enclosed kanR sequence in between the telomere sequences.

pREP-his3$^+$

The *his3*$^+$ gene was cloned into pREP3X using PstI and SacI sites to generate pREP-*his3*$^+$.

pREP42-taz1$^+$

pREP42- *taz1*$^+$ was used in the construction of the taz1+ o/e strain. The *taz1*$^+$ORF was cloned in a pTOPO vector (Invitrogen). pTOPO was then digested with BamHI and SalI and the resulting *taz1*$^+$fragment was cloned into the BamHI and SalI sites of pREP42 vector.

Media and genetic methods

We used standard recipes for Yeast Extract with supplements (YES) and Edinburgh minimal medium (EMM) as described in (27). Methods of transformation, sporulation and tetrad dissection are described in (27–29).

Strains

Strains used in this study are described in Supplementary Table S1. Lines derived from genetic crosses are indicated in the parental column with both parental strains indicated. All deletions were performed using the procedures described in (28).

taz1$^+$ *o/e*

To create strain MGF1898, pREP42-*taz1*$^+$ was digested with KpnI and transformed for integration. Insertion at the *taz1*$^+$ locus was confirmed by PCR.

Primers

Primers used for amplification and sequencing of captured fusions were as follows: 495:GAACTTCAGCCTTATCG CTG; 496: CCACGGAAATAACCGAACCA; 613: GGG TAATAATTGATATGAGGGC; 614: CCACGGAAAT AACCGAACCA. Primers used for confirmation of insertion of pREP42-*taz1*$^+$ fragment at the *taz1*$^+$ locus were as follows: 178: TTCGCCTCGACATCATCTGC; 375: CCTCAGTGGCAAATCCTAAC; 711: TCTTTTACAG TTTCCTTCCC; 717:ATTGCAGAGTAAACACGACG; 736: TGGACTTTGCGTATGAGACG.

Serial dilution assays

Cultures were grown at 32°C to logarithmic phase and re-suspended to a cell density of 1×10^7 cells/ml. Ten-fold serial dilutions were performed in EMM media, and 5 µl of each dilution were spotted onto EMM plates with the described supplements and incubated at 32°C up to 7 days.

Telomere fusion assays

Cultures were grown at 32°C to logarithmic phase in EMM media containing Leucine and plated on solid EMM media containing Leucine ± Histidine. Plates were incubated at 32°C up to 7 days and colonies were counted. For time course experiments, cultures were diluted at each time point to 5×10^5 cells/ml and incubated the appropriate times in EMM media containing Leucine ± Histidine.

Western blotting

Cells were lysed in 20% TCA and the resulting protein extracts were resolved by SDS-PAGE, transferred to PVDF membranes (GE Healthcare RPN203D) and probed with primary α-Taz1 antibody (a gift from Julia P. Cooper). After incubation with HRP-conjugated secondary antibodies (GE Healthcare NA934), bands were visualized using ECLPlus (GE Healthcare RPN2132) in a STORM scanner (Amersham). Quantification was performed using ImageJ software.

Southern blotting

Genomic DNA was obtained from exponentially growing cells in YES or EMM media with supplements, using the phenolic extraction method described (27) and digested with the appropriate restriction enzymes. Southern blot analysis was performed as described (10). Briefly, DNA was separated in 0.8% agarose gels and transferred by capillarity to genomic blotting membranes (Bio-Rad, #162-0196). The DNA was then cross-linked using UV radiation and the membranes were hybridized using Church–Gilbert solution at 65°C. An overnight incubation was performed with a telomere repeat probe or a *rad4*$^{+}$ genomic probe labelled with ^{32}P using the Prime-it II random primer labeling kit (Stratagene). The membrane was washed for 30 min at 65°C with a 1× SSC 1% SDS solution and exposed to a PhosphoImager screen (Amersham) for 1–3 days depending on signal strength. The PhosphoImager screen was scanned with a STORM scanner (Amersham). Telomere length was calculated by normalizing molecular weight with telomere signal intensity, as described in (30). Number of pFT2 copies per cell was calculated by dividing signal intensity from *ars1* fragment present in pFT2 with the signal intensity from the endogenous *ars1*. This number was multiplied by a factor of 0.3 to account for the measured fraction of cells in a population under selection for *LEU2* harbouring pFT2 (See Supplementary Figure S2).

PCR reactions

Mini-chromosome end fusions were amplified by PCR. Forward primers (495: GAACTTCAGCCTTATCGC TG; 613:GGGTAATAATTGATATGAGGGC) were used for promoter proximal sequencing and the reverse primers (496:CCACGGAAATAACCGAACCA; 614:CCACGGAAATAACCGAACCA) for terminator proximal sequencing.

RESULTS

Direct assay to capture telomere fusions

To devise a scheme to detect telomere fusions, we resorted to the ability of introns to bear gene-unrelated sequences, such as telomeres. We cloned the *his3*$^{+}$ gene in a fission yeast plasmid carrying a *LEU2* marker, allowing for double selection in media lacking both histidine and leucine. Subsequently, we introduced two telomeres of 258 bp in a head to head arrangement in a unique site of the second intron of *his3*$^{+}$ thus creating pFT2 (Figure 1A). These telomeres were flanked by 80 bp of subtelomeric sequences on each side. The telomere sequences did not impair *his3*$^{+}$ expression and allowed for growth in media lacking both leucine and histidine (pFT2-Cir, Figure 1B). We then linearized pFT2 between the telomere sequences and transformed WT cells, thus creating a mini-chromosome in which the *his3*$^{+}$ gene was split between the two extremities of the plasmid (Figure 1A). Consequently, the linear mini-chromosome pFT2 could be maintained through selection using *LEU2* expression but was now unable to grow on media lacking histidine (pFT2-Lin, Figure 1B). We reasoned that any event that would disrupt telomere protection leading to plasmid end joining would restore the initial circular configuration of pFT2 and allow for *his3*$^{+}$ expression. We also asserted that intron length would not be limiting in our assay by observing the unimpaired growth of strains harbouring circular pFT2 carrying a 1.3 kbp Kan^{r} cassete in the *his3*$^{+}$ intron (data not shown). As long as the *his3*$^{+}$-coding sequence is not eroded, this system identifies any type of end joining to occur between the ends of the mini-chromosome, as introns do not rely on a coding reading frame.

We next gathered evidence that the mini-chromosome was propagated linearly in cells and that telomeres were functional. We produced genomic DNA from fission yeast cells and digested it with either EcoRV that would release the *his3*$^{+}$ promoter proximal telomere (T Prom) or XhoI that cuts near the *his3*$^{+}$ terminator, generating a fragment containing the terminator proximal telomere (T Term; Supplementary Figure S1). We next performed Southern blotting using a telomere probe that revealed the telomere sequences present in the cell's chromosomes and in the newly generated mini-chromosome (Figure 1C). Both chromosome ends showed a distribution of sizes typical of telomeres, suggesting that the ends of the mini-chromosome were free and were being used as a substrate by telomerase.

To test the mini-chromosome stability and to confirm that it was independent of the remaining chromosomes, we analysed the ability of these cells to lose pFT2 in either the linear or the circular form. Cells were grown in media containing leucine and histidine for several generations and then tested for the ability to retain pFT2. Fission yeast plasmids lack centromeric sequences and are thus missegregated and lost when not under selection (31). We confirmed that pFT2 either in the circular or linear configuration were lost at a similar rate as other plasmids (Supplementary Figure S2). Thus, our mini-chromosome was maintained independently of the remaining genome, showing that its telomeres were able to recruit telomerase and protect its ends.

We wanted to know whether we could use our assay to analyse chromosome-end fusions. As a proof of principle, we crossed an early generation telomerase mutant with the strain harbouring the pFT2 mini-chromosome and allowed the progeny to undergo telomere erosion over several days. On each time point, we collected samples for Southern blotting and plated cells on media containing and lacking histidine to measure the ability to fuse the ends of the mini-chromosome. Using a telomere probe, we could observe the slow erosion of telomeres in *trt1*Δ mutants over several days (Figure 1D). Throughout this experiment, control WT did not generate detectable colonies in histidine-depleted media (Figure 1E). However, by day 6, coincident with the lowest signal for the mini-chromosome telomeres, *trt1*Δ cells showed a steadily increase in *his3*$^{+}$-expressing cells. Over the course of the experiment, productive mini-chromosome fusions increased to represent 5–8% of all the cells harbouring the pFT2 plasmid. Given that several mini-chromosome fusions are likely to occur in a way

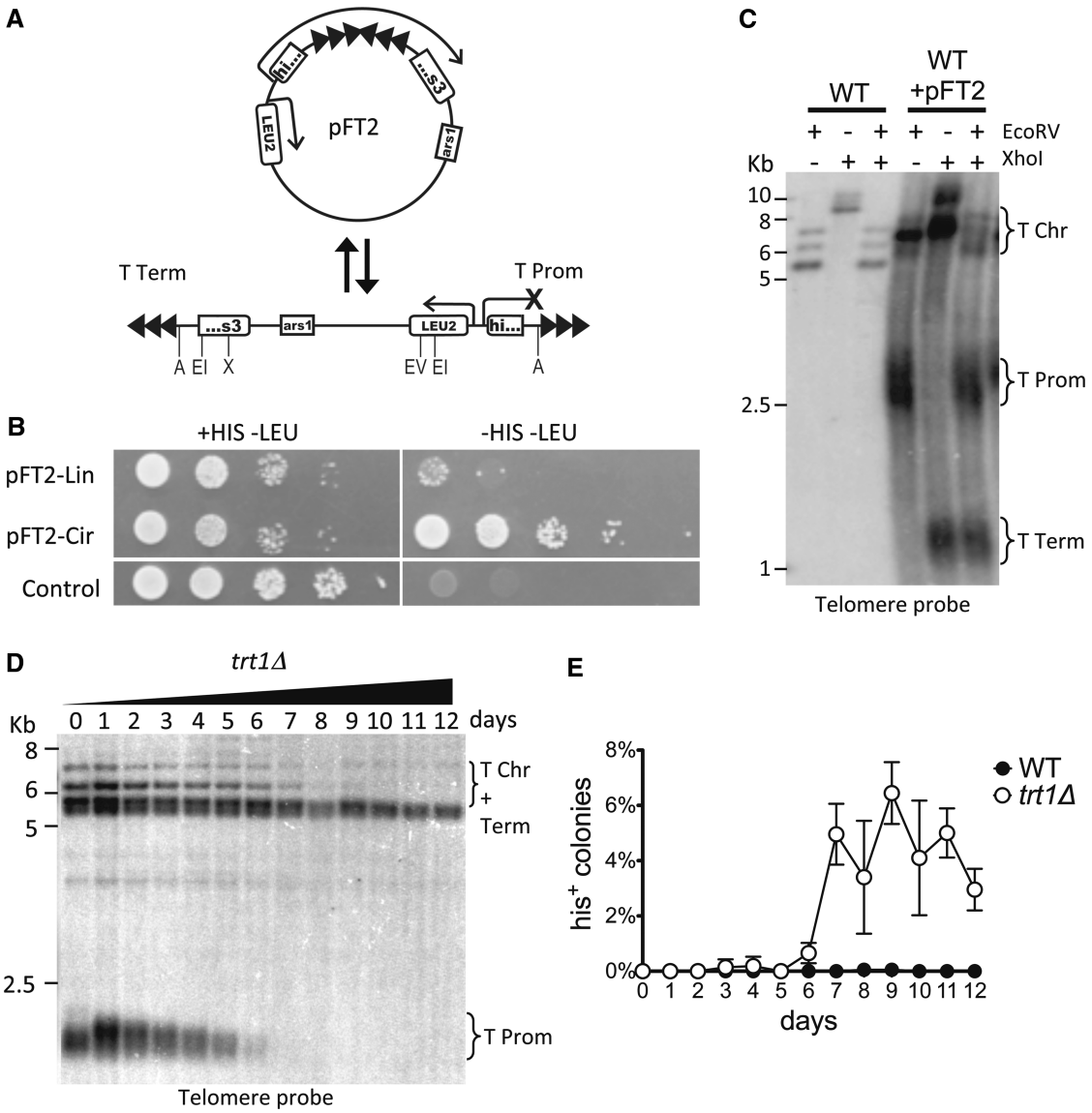

Figure 1. Mini-chromosome assay used to detect telomere fusions. (**A**) Fusion of both ends of the linear pFT2 mini-chromosome generates a circular plasmid that expresses the *his3*$^{+}$ gene. Relevant restriction sites are indicated as follows: A—ApaI; EI—EcoRI; EV—EcoRV; X—XhoI. (**B**) Ten-fold serial dilutions of WT cells carrying either the linear (pFT2-Lin) or circular (pFT2-Cir) mini-chromosome plated on permissive (+HIS −LEU) or restrictive (−HIS −LEU) media. Control cells contain a *LEU2*-based plasmid that lacks the *his3*$^{+}$ gene. (**C**) Southern blot analysis of telomeres in a WT strain harbouring pFT2. Genomic DNA extracts were digested with EcoRV and/or XhoI enzymes and probed for telomeres (see Supplementary Figure S1 for details). Promoter-proximal (T Prom), terminator-proximal telomeres (T Term) and chromosomal telomeres (T Chr) are depicted. (**D**) Southern blot analysis of telomere shortening in a *trt1*Δ strain. Cells were grown in liquid culture and genomic DNA extracts were digested with EcoRV. (**E**) Frequency of WT and *trt1*Δ strains able to express histidine. Percentages were calculated by dividing the number of colonies growing in +HIS −LEU and −HIS −LEU. Error bars represent SEM of three replicates for each time point.

that fail to reconstitute *his3*$^{+}$ expression, we anticipate that we are under-estimating the whole population of fusions.

Taz1 levels are limiting to control telomere length

We wanted to define the impact for the cell to harbour more telomeres than the ones it usually carries. Haploid fission yeast contains three chromosomes and spends most of its cell cycle in S/G2 phases. Thus, cells carry a maximum of 12 telomeres at any given time. Introducing several other telomeres could alter telomere dynamics. To investigate the impact of extra telomeres per cell, we first quantified how many mini-chromosomes would a cell carry on average. We used Southern blot analysis to quantify the *ars1* fragments, which are present as a single copy both in the genome and in the mini-chromosome (Supplementary Figure S3). Given that not all cells in the population carry a mini-chromosome due to natural plasmid loss, even when under selection, we calculated that the mini-chromosomes would be in a range of 5–7 copies per cell, thus increasing the overall number of telomeres.

To assess the impact of extra copies of telomeres, we looked at telomere length in cells carrying the

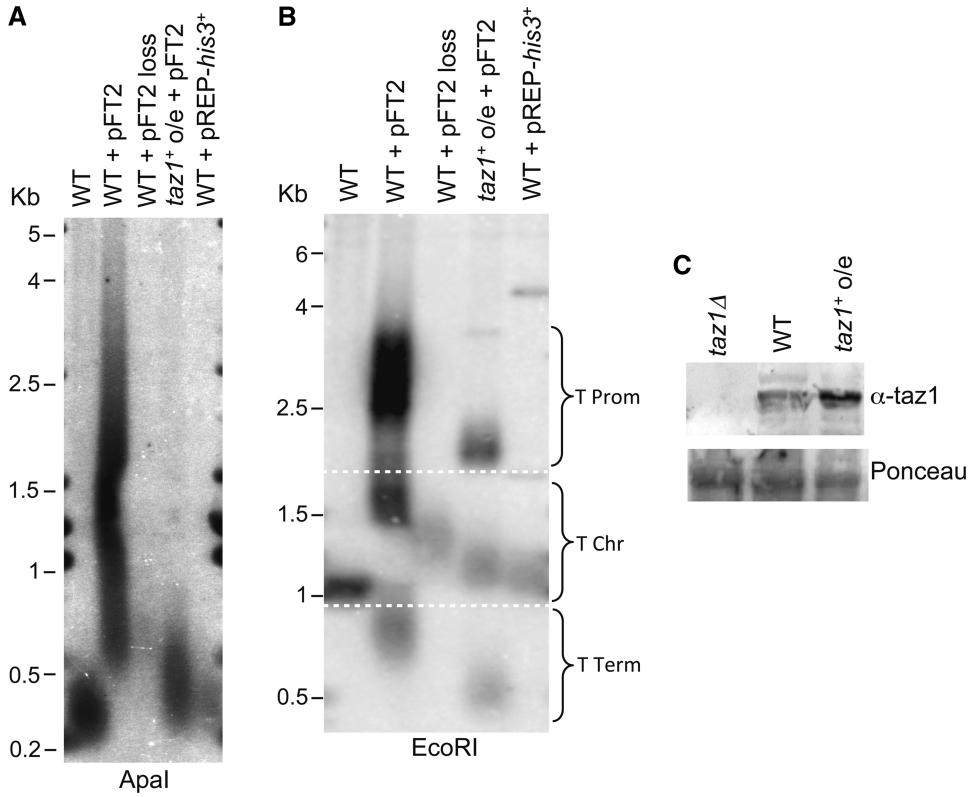

Figure 2. Linear mini-chromosomes affect telomere length through dilution of Taz1 (**A**) Southern blot analysis of telomere length in WT strains carrying pFT2, after pFT2 loss and with taz1$^+$ o/e. Genomic DNA extracts were digested with ApaI and probed for telomere DNA. ApaI digestion does not discriminate between endogenous and pFT2 telomere ends. (**B**) The same genomic DNA extracts analysed in (A) were digested with EcoRI and probed for telomere DNA. EcoRI digests separate the chromosomal telomeres (≈1 Kb) from the mini-chromosome telomeres. (**C**) Over-expression of Taz1 was monitored on a western blot using α-taz1 (top) and Ponceau staining as loading control (bottom).

mini-chromosome and compared to control cells. Using an ApaI restriction enzyme that is unable to discriminate the source of the telomeres analysed, we observed that the presence of the mini-chromosome gives rise to a 3- to 5-fold increase in overall telomere length (Figure 2A, lane 2). We then used an EcoRI digest to discriminate between the chromosome telomeres and those harboured in the mini-chromosome. Southern blots verified that the chromosome telomeres were elongated in cells carrying the mini-chromosome (Figure 2B, lane 2). The mini-chromosome was the cause for the changes in telomere length, since telomere length decreased in cells that lost the linear plasmid (Figure 2B, lane 3). The same increase was observed in a strain carrying a circular configuration of the mini-chromosome (Supplementary Figure S4B). Thus, as a consequence of increasing the number of telomeres in the cell, the overall telomere length is increased, suggesting the titration of a limiting factor that controls telomere length.

We aimed at having a plasmid fusion assay that mimics WT cells as close as possible. Even though there is no indication that small variation in length, such as the one observed, results in deficiencies in telomere protection, we set up to identify the possible regulator of telomere length that was limiting. Using plasmids carrying the *nmt1*$^+$ promoter, we independently over-expressed Taz1, Rap1, Pot1 and Ccq1 in cells carrying the mini-chromosome (data not shown). Only Taz1 over-expression resulted in an almost complete reduction of telomere length to wild-type length of all telomeres. The same result was observed when we integrated a Taz1 over-expression cassette in the genome of a strain, to which we called *taz1*$^+$ *o/e* (Figure 2B). This strain was used in all remaining experiments. By western blotting, we estimated that Taz1 was over-expressed about 2-fold when compared with WT levels (Figure 2C). Thus, levels of the telomere binding Taz1 are limiting in fission yeast and, when telomere number increases, this results in net telomere elongation. Accordingly, budding yeast's telomere-binding factor Rap1 was also limiting upon increase in telomere numbers (32,33).

*trt1*Δ mini-chromosome fusions are MRN dependent and occur via SSA/MMEJ repair

Capturing telomere fusions residing on a plasmid allows us to study not only their abundance but also the mechanism whereby they arise. Previous studies revealed that chromosome-end fusions in *trt1*Δ in fission yeast were a consequence of the *rad16*$^+$ (*ScRAD1* and mammalian XPF)-dependent SSA/MMEJ pathway (18). To test the genetic requirements for mini-chromosome fusions, we produced *trt1*Δ mutants in which we deleted specific key factors regulating different DNA repair pathways. We allowed *trt1*Δ, *trt1*Δ *rad16*Δ and *trt1*Δ *lig4*Δ mutants

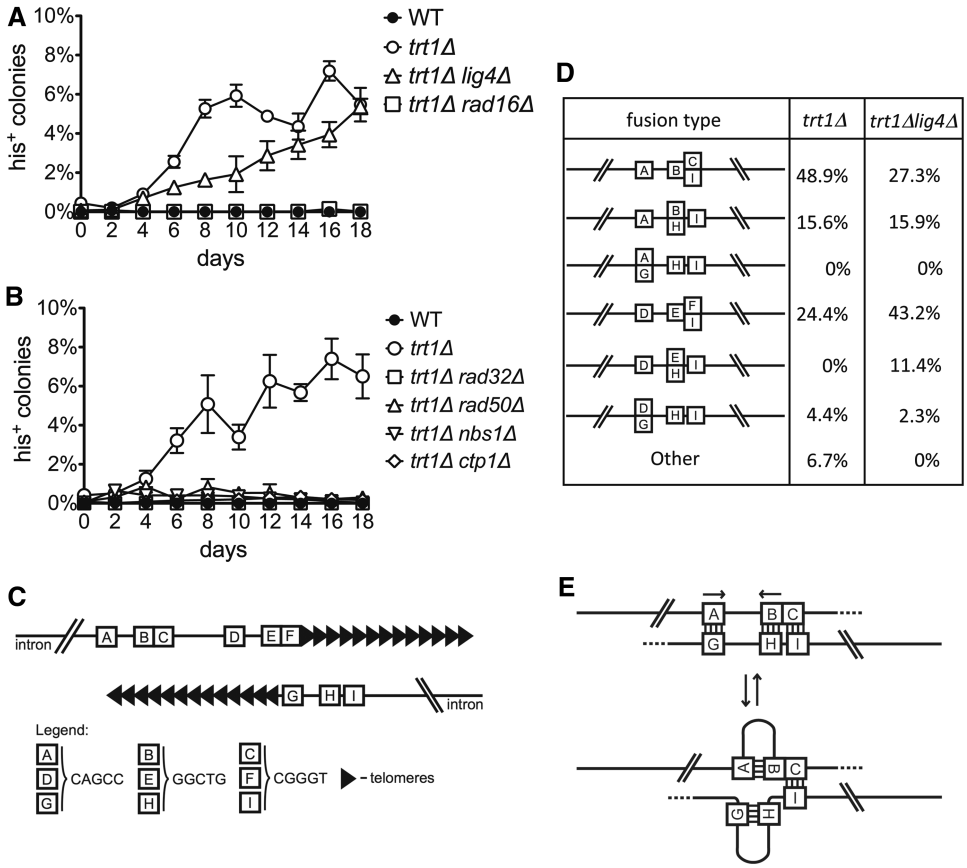

Figure 3. Mini-chromosome fusions in *trt1*Δ occur via *rad16*-dependent SSA/MMEJ repair and require MRN/Ctp1. (**A**) Expression of histidine during telomere erosion in *trt1*Δ, *trt1*Δ *lig4*Δ and trt1Δ *rad16*Δ strains. (**B**) Expression of histidine during telomere erosion in *trt1*Δ, *trt1*Δ *MRN*Δ and *trt1*Δ *ctp1*Δ strains. Cells were plated daily on +HIS −LEU and −HIS −LEU media. Frequencies for (A) and (B) were calculated by dividing the number of colonies growing in +HIS −LEU and −HIS −LEU. Error bars represent SEM of three replicates for each time point. (**C**) Schematic representation of the 5 nt microhomologies found at the fusion junctions in *trt1*Δ and *trt1*Δ *lig4*Δ strains. (**D**) Frequency of each microhomology at fusion junctions. *trt1*Δ strain, $n = 45$; *trt1*Δ *lig4*Δ strain, $n = 44$. (**E**) A model for microhomology-mediated fusions in *trt1*Δ and *trt1*Δ *lig4*Δ strains.

carrying the mini-chromosome to undergo telomere erosion and quantified the number of colonies expressing histidine as a measure of end-joining events. Both *trt1*Δ and *trt1*Δ *lig4*Δ mutants produced substantial mini-chromosome fusions (Figure 3A). This was consistent with previous observations (34), in which Lig4-dependent NHEJ repair was dispensable for generating *trt1*Δ survivors in fission yeast. In contrast, *rad16*$^+$ was required for *trt1*Δ mini-chromosome end fusions (Figure 3A). These results demonstrate not only that the SSA/MMEJ pathway is required for joining the ends of the mini-chromosome, but also that the repair mechanisms triggered on chromosomes are the ones responsible for repairing the ends present in the mini-chromosome, thus validating our assay.

In parallel, we investigated the role of MRN (Rad32/Rad50/Nbs1)/Ctp1 (MRX/*ScSAE2* or mammalian MRN/CtIP) in generating mini-chromosome end fusions in *trt1*Δ mutants. For this purpose, we established mutants of each component of this complex and conjugated them in a *trt1*Δ background. Absence of MRN/Ctp1 also failed to produce *trt1*Δ survivors capable of expressing histidine (Figure 3B). Thus, MRN and its nuclease partner Ctp1 are required to process mini-chromosome ends for the SSA/MMEJ repair reaction.

In contrast to TRF2 and *taz1*Δ mutants, chromosome-end fusions in *trt1*Δ mutants occurred only after total telomere erosion. We set out to investigate the sequences present at the junction of the mini-chromosome. We devised PCR reactions with primers on each side of the intron and verified that all of the *trt1*Δ histidine-producing cells possessed an intact *his3*$^+$ intron ($n = 45$), denoting that they occurred between the cloned subtelomeric sequences of the mini-chromosome. We sequenced *trt1*Δ and *trt1*Δ *lig4*Δ fusion junctions and, to our surprise, >90% involved a set of three pentanucleotide G-rich sequences present within the 85 bp subtelomeric region (Figure 3C and D). Consistent to what was previously reported (3,20,22), all but one of the *trt1*Δ fusions involved telomere sequences. The pentanucleotide sequences were arranged in a direct repeat spanning 30 bp and were present at both ends of the mini-chromosome (Figure 3C, Supplementary Figure S5), two promoter proximal (depicted as ABC and DEF) and one terminator proximal (GHI).

Not all pentanucleotides were equally involved in fusions, and there was a clear preference for those with C/I and F/I pairs (73.3% and 70.5% in *trt1*Δ and *trt1*Δ *lig4*Δ, respectively, Figure 3D). We wondered why fusions comprising the pentanucleotide present in C, F and I were

dominant. We realized that the remaining other two pentanucleotides formed an inverted repeat spaced by 14 bp that could be engaged in a hairpin structure (Figure 3E). Conceivably, as telomeres erode, ssDNA is generated at subtelomeric regions of the mini-chromosome creating the opportunity for secondary DNA structures such as hairpins. This may momentarily stabilize the sequences involved in the hairpin, thus exposing the adjacent pentanucleotide for SSA/MMEJ repair. This model is consistent with the requirement of MRN/Ctp1 in *trt1*Δ fusions, since the complex would be engaged in processing the hairpin as part of the DNA repair reaction (35,36).

Telomere–telomere fusions occur in unperturbed WT cells via NHEJ repair

As we generated our telomere fusion assay, we wondered about the rare colonies appearing in unperturbed WT cells grown on media lacking histidine (Figure 1B). These escapers originated from previous cells that carried the linear mini-chromosome and did not express *his3*$^+$, suggesting that they were the outcome of unsuspected fusions taking place in unperturbed cells. We measured the frequency of these events. In *taz1*$^+$ *o/e* cells, the frequency of colonies expressing *his3*$^+$ was $1.4 \times 10^{-4} \pm 0.5 \times 10^{-4}$ (SEM, $n = 3$; Figure 4A). In contrast, cells with longer telomeres in which Taz1 was limiting had a higher reversion frequency of $3.4 \times 10^{-4} \pm 0.9 \times 10^{-4}$ (SEM, $n = 3$), suggesting that insufficient Taz1 levels to maintain normal telomere length also resulted in reduced protection from end fusions.

Chromosome-end fusions in unperturbed cells expressing telomerase were previously reported in transformed human cell lines (23). These were the result of completely eroded telomeres fusing to a shorter telomere involving microhomologies at the junction. To identify the mechanism behind our mini-chromosome fusions, we first investigated the genetic requirements underlying these events. As previously, we deleted the genes involved in cells containing the mini-chromosome and quantified the ability of expressing the *his3*$^+$ reporter gene. In contrast to the observed mini-chromosome fusions in *trt1*Δ survivors, *rad16*$^+$-dependent SSA/MMEJ repair is dispensable in *taz1*$^+$ o/e (Figure 4A). However, mutations in key elements of the NHEJ pathway, such as *pku70*Δ and *lig4*Δ, reduced the number of *his3*$^+$-expressing colonies about 10-fold (Figure 4A). These results indicate that end-fusions occurring in unperturbed cells are processed by a distinct pathway to the ones caused by gradual telomere erosion, suggesting that they are fundamentally different uncapping events.

We next asked whether MRN/Ctp1 was required for NHEJ-mediated fusions of mini-chromosome ends. In contrast to budding yeast, MRN is dispensable for NHEJ repair in *S. pombe* as measured on plasmid-based assays (37). Surprisingly, mutants in all subunits of MRN, including Ctp1, were deficient in joining the two ends of the mini-chromosome by NHEJ repair (Figure 4B). Our data suggests that, in contrast to NHEJ measured in naked plasmid ends, MRN/Ctp1 is required to process mini-chromosome ends before undergoing end-joining reactions.

The requirement of NHEJ repair for chromosome fusions in unperturbed cells suggested that a different event occurred at the ends of our mini-chromosome. To attempt at identifying the incident that originated the end-fusion, we sequenced the junction at *his3*$^+$ mini-chromosomes. Almost all fusion events involved two telomeres on each side of the junction (89.3%, $n = 28$; Figure 4C and D and Supplementary Figure S6). The remaining involved one telomere and a subtelomere or two subtelomeres (7.1% and 3.6%, respectively). In all cases, we did not observe clear microhomologies at the junction, even though fusions often involve guanidine bases, as expected from G-rich telomere sequences (data not shown). Thus, in contrast to the previous studies in immortalized humans cell lines (23), our data suggest that uncapping events occur at telomere sequences that result in end-fusions via NHEJ repair.

Telomere fusions occur between half-sized telomeres

Even though mini-chromosome fusions exhibited substantial telomere sequences in a head to head arrangement, it was not clear whether the end-joining event resulted from one of them being too short to sustain telomere protection. To investigate the telomere length distribution in fusions, we organized telomeres in a length histogram (Figure 5A). There was a 2-fold size difference between the median telomere size at T Prom and T Term (T Prom 232 bp and T Term 111 bp; interquartile range (IQR) 92–291 bp and 29–147 bp, respectively). This was consistent with the average telomere lengths exhibited by the mini-chromosome, as analysed by Southern blot (T Prom 415 bp and T Term 184 bp, Figure 2A and B). Interestingly, a 2-fold difference between the size of T Term and T prom was also observed for the strain with deficient Taz1 levels (Figure 2B, lane 2). Thus, even though the methods used provide measures of different accuracy, our data suggest that most fusions occur between telomeres of about half the normal length observed in the linear configuration.

The difference in length between the promoter and terminator telomeres could arise due to a telomerase preference for one telomere over the other. This preference may reflect transcription of both the T Prom and endogenous telomeres, either through *his3*$^+$ or TERRA transcription, which does not occur at the T Term telomere. To discriminate whether one telomere was more frequently engaged by telomerase over the other, we compared the degenerated telomere sequences of fission yeast present at fusion junctions. As in most yeast species, *S. pombe* telomerase is 'faulty' providing other nucleotides in between the consensus $(GGTTAC)_n$. This property allowed us to recognize the original telomere sequence and identify how much erosion telomeres suffered and which new sequences were added (Figure 5B). We suggest that telomerase has a preference for T Prom since it had added repeats to most telomeres engaged in fusions (26/28). In contrast, only about half of the T Term telomeres have new sequences added (16/28). As a result,

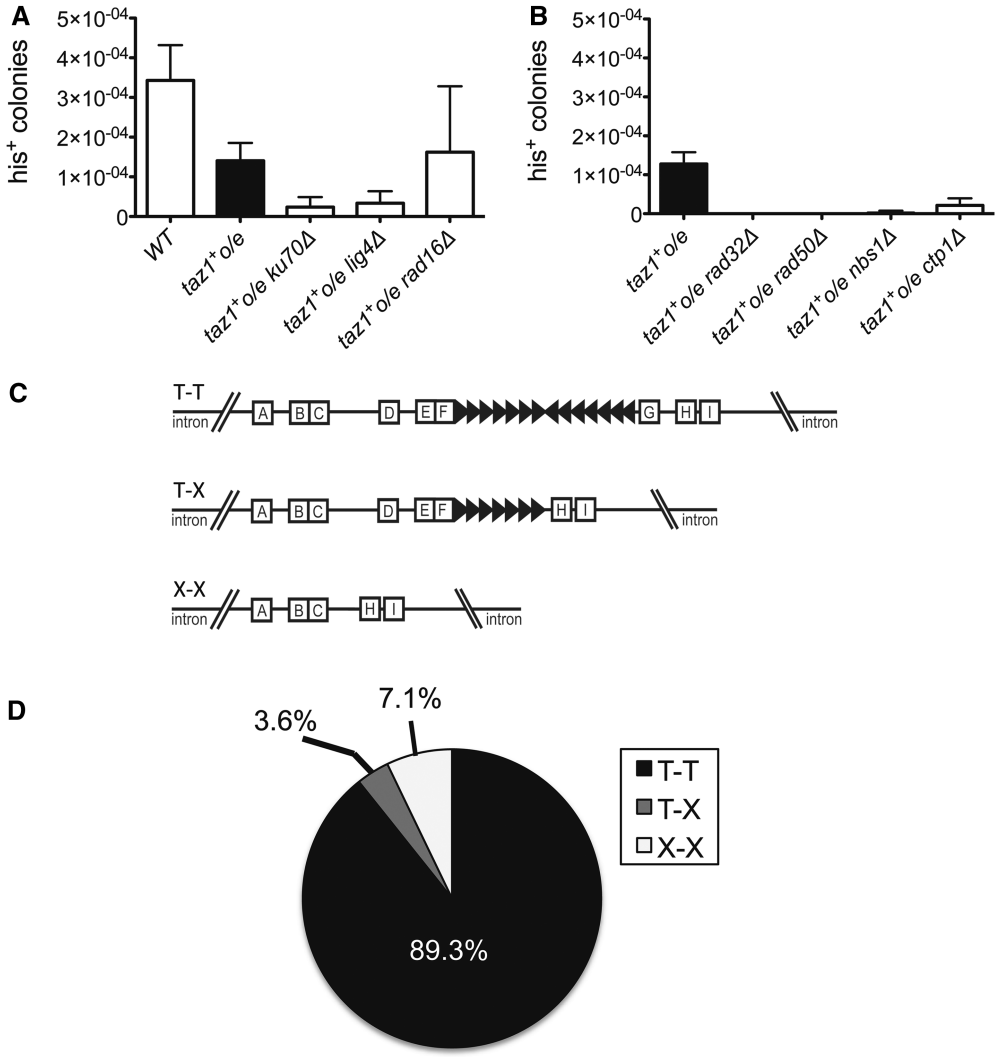

Figure 4. Spontaneous telomere fusions occur in unperturbed WT cells. (**A**) Frequency of fusions in WT, and *taz1*$^{+}$*o/e ku70*Δ, *taz1*$^{+}$*o/e lig4*Δ and *taz1*$^{+}$*o/e rad16*Δ strains. (**B**) Frequency of fusions in *taz1*$^{+}$*o/e rad32*Δ, *taz1*$^{+}$*o/e rad50*Δ, *taz1*$^{+}$*o/e nabs1*Δ and *taz1*$^{+}$*o/e ctp1*Δ strains. In (A) and (B), cells were plated in +HIS −LEU and −HIS −LEU media in triplicate. Percentage of his+ cells in the population was determined by dividing the number of his+ colonies by the total number of colonies. Error bars represent SEM of three independent experiments. The threshold of detection for (A) and (B) is 1×10^{-5} his+ colonies/total sample. (**C**) Classification of telomere fusion types. T-T (telomere to telomere): fusion junctions containing telomeric DNA from both ends. T-X: fusion junctions containing telomeric DNA on one end fused to a subtelomeric region. X-X: fusion junctions containing non-telomeric DNA. (**D**) Frequency of T-T, T-X and X-X fusions.

telomere shortening is more evident on terminator than on promoter proximal telomeres (Figure 5B). Median length of original telomere was half as long on the terminator proximal telomere (T Prom 101 bp and T Term 68 bp; IQR 75–149 bp and 18–103 bp, respectively). These differences could be explained by different affinity for telomerase, such that the T Term telomere is elongated only half of the times as the T Prom and thus erodes further acquiring a smaller steady state length.

Why are telomeres engaging in end-joining reactions? We reasoned that if one telomere would be critically short, it would engage in fusions with other telomeres independently of their size. Such was observed in budding yeast for fusions between a DSB and telomeres (24). However, this was not the case for our assay. By plotting telomere length of T Prom against T Term, we observed a positive correlation of sizes such that the longer the telomere was on one side, the longer it was on the other ($R^2 = 0.62$, $n = 28$; Figure 5C). Thus, the fusions observed cannot be explained by critical telomere shortening at one end of the mini-chromosome. Instead, telomeres appear to become stochastically deprotected, thereby undergoing NHEJ repair with neighbour telomeres.

DISCUSSION

Telomere erosion leading to critically short telomeres has been widely implicated in cancer (23,38–42). Studies using primary human cell lines have shown that telomeres engage in end fusions upon continuous erosion (23). Here, we show that unperturbed cells with seemingly functional telomeres can also engage in chromosome-end fusions. Telomere-to-telomere fusions are an undervalued

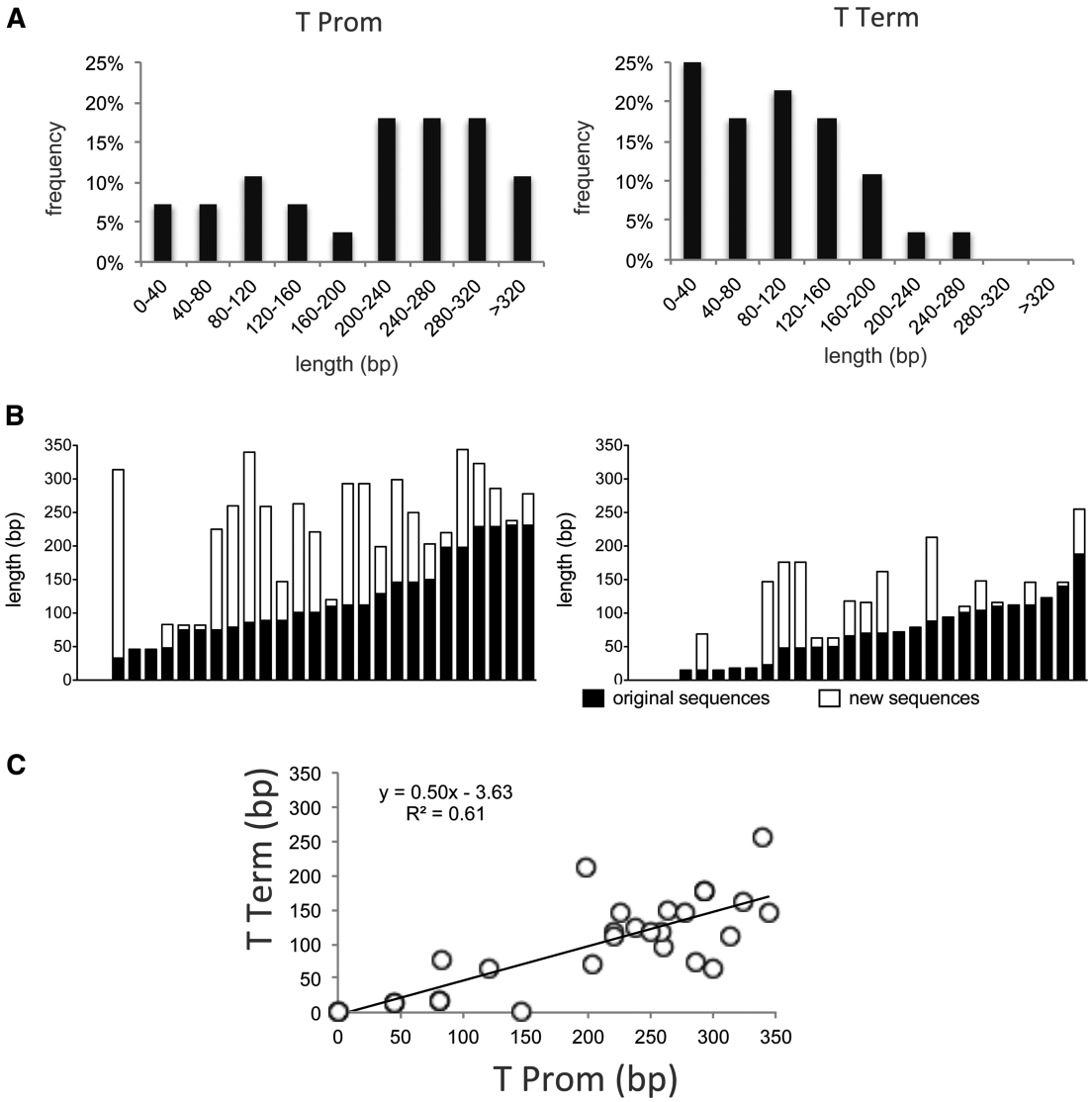

Figure 5. Mini-chromosomes undergo end fusions at half-sized telomere length. (**A**) Frequency of telomere fusions according to telomere length, at the promoter-proximal telomere (T Prom) and at the terminator-proximal telomere (T Term). (**B**) Telomere size of each telomere ($n = 28$). White bars represent originally cloned telomere sequences and black bars represent sequences added *in vivo*. (**C**) Pairwise telomere length distribution of mini-chromosome telomeres involved in fusions.

phenomenon owing to the difficulties in capturing and sequencing these events. This partially stems from the lack of quantitative positive detection assays (43–46). One such assay using an induced DSB in budding yeast provided an extremely low frequency of telomere-to-DSB fusion events [8.4×10^{-8} (24)]. Since we found far more frequent rate of telomere-to-telomere fusions (1.4×10^{-4}), it is tempting to speculate that spontaneous chromosome fusions may be a relevant trigger of genome instability and carcinogenesis. Consistent with this idea, recent evidence show telomere-to-telomere fusions in human breast carcinoma (42). The disparity in frequencies between our result and the one previously reported may simply reflect differences in the experimental setup. Unlike our assay, the telomere-to-DSB fusion assay relies on coinciding a DSB with transient telomere deprotection. Since G1 phase is predominant in budding yeast asynchronous cultures, a DSB generated during this stage may not be available in late S phase when telomeres are replicated and possibly deprotected. In contrast, our assay relies exclusively on transient telomere deprotection and these events occur synchronously by their own nature. Alternatively, the higher frequency of telomere fusions measured in our assay may simply reflect the artificial nature of the reporter construct. Our mini-chromosome possesses considerably short subtelomeric regions (ca. 80 bp) in comparison with chromosomal subtelomeres that encompass 10 kbp. Moreover, telomeres were introduced within an intron of a transcribed gene, a feature that may affect telomere protection.

The frequency of telomere fusions measured in our assay is likely to under-represent the total amount of those present in WT and *trt1*Δ mutants. Our system is unable to detect unproductive mini-chromosome-end

fusions, i.e. all those that fail to regenerate *his3*$^+$ expression. These could involve (i) the destruction of the coding sequence or intronic splice sites of the reporter gene; (ii) fusions between mini-chromosome and endogenous chromosome ends; (iii) fusions between mini-chromosomes that do not pair opposite ends or even (iv) the generation of linear *trt1*Δ survivors that continuously undergo homologous recombination (HR) at the chromosome ends.

Telomere-to-telomere fusions require the NHEJ pathway similar to what was found in *taz1*Δ, *rap1*$^-$ and TRF2$^{F/-}$ mutants (11,14,47,48). They also depend on the MRN complex, as was observed in TRF2$^{F/-}$ and *taz1*Δ fusions (49,50). Because in addition to MRN, fusions require the Ctp1 nuclease that is not present during G1 (51), these events are likely to occur later in the cell cycle, perhaps during DNA replication when telomeres are exposed. In fission yeast, *taz1*Δ telomeres are subject to HR during S/G2 phase of the cell cycle (52). However, dysfunctional telomeres can undergo NHEJ-mediated fusions in S/G2 when HR is compromised (11). We anticipate that upon DNA replication, telomere protection may occasionally be faulty. In late S phase, telomeres unfold to accommodate the DNA replication machinery causing momentary deprotection (53). This is corroborated by DNA damage responses being initiated every S phase as the replication fork reaches chromosome ends (16,54). Taz1 levels may also be limiting during this period to protect the newly duplicated telomeres. In support of this hypothesis, we observed that a strain with insufficient Taz1 undergoes telomere fusions at a higher frequency.

In contrast to unperturbed cells, chromosome fusions in the absence of telomerase were mediated by SSA/MMEJ repair. Choice of DSB repair may simply reflect telomere length and sequence. Although shelterin-protected longer telomeres prevent DNA-end 5′-resection required for SSA/MMEJ repair (55), ssDNA generated at critically short telomere inhibits NHEJ repair (49). Additionally, microhomologies are largely absent from telomere sequences involved in end-joining reactions [e.g. 5′-(TTAGGG)$_n$/(CCCTAA)$_n$-3′]. Previous work in *pot1*Δ and *trt1*Δ mutants showed extensive degradation of chromosome ends up to 13 kb (20). In contrast, our mini-chromosome ends in *trt1*Δ mutants fuse at specific pentanucleotide microhomologies contiguous to telomeres but not within the juxtaposed intronic region of *his3*$^+$, suggesting that intron sequences may be refractory to end-joining reaction. We observed that microhomologies were arranged in inverted repeats that, when resected, could fold back and engage in DNA hairpin formation. Fusions in *trt1*Δ mutants require the MRN complex and Ctp1. Thus, hairpins may serve as stabilizing structures for eroding DNA ends, which are subsequently processed by MRN/Ctp1 and captured in end-fusion reactions (36,56). MRN was similarly required for chromosome fusions involving critically short telomeres in Arabidopsis and human cells (45,57), and telomerase RNA mutants in budding yeast (58). Alternatively, hairpins formed during DNA replication can lead to end-joining reactions (59,60). Recent evidence shows that DNA replication plays a role in chromosome-end fusions in *Caenorhabditis elegans* (61). However, we were unable to detect fusions involving pentanucleotide repeats in WT cells, suggesting they require telomere resection prior to fusion. Future studies will reveal the role of these naturally occurring subtelomeric DNA microhomologies in telomere fusions.

Our mini-chromosome allowed us to assess end-to-end fusions in several distinct genetic backgrounds. Furthermore, we established that there are two distinct pathways that generate chromosome-end fusions, which are determined by the mechanism of uncapping, and that both are likely to be of importance in the development of genome instability and cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–6.

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*“I **drowned** the last whale. I can do **anything** now.”*

— Hank “Beast” McCoy, possessed by the Sublime entity, in Grant Morrison’s and Marc Silvestri’s *New X-Men* #154